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Studies of Heinz Body Formation*

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The hemolytic anemias with Heinz-body formation following exposure to a great variety of drugs have been described by many investigators. These erythrocyte inclusion bodies have also been demonstrated in certain types of congenital hemolytic anemia with abnormal pigment metabolism (1 2) following splenectomy (3 4) and in hypoplasia or agenesis of the spleen (5)

Spontaneous appearance of Heinz bodies has been reported in newborn infants with a greater incidence in the premature, (6 7 8, 9) This phenomenon, which may be associated with hemolytic anemia, disappears within the first weeks of life. Sansone described a marked susceptibility of the newborn blood to develop these inclusion bodies in the presence of acetylphenylhydrazine (9)

The objective of the present study is to demonstrate *in vitro* the factors controlling Heinz body formation and to compare this in the blood of prematures and normal adults with and without exposure to toxins.

Materials and Methods

To demonstrate Heinz bodies, both wet and dry preparations were made with either crystal violet (2% in normal saline) or methyl violet (0.5% in saline) For electron microscopy samples were placed into 1 to 2% osmium tetroxide with s-collidine buffer according to Bennett and Luft (10) or with phosphate buffer After dehydration in ethanol, all samples were imbedded in EPON 812 according to Luft (11) Different hardness was used depending on the material. For fresh untreated red cells, harder EPON was used Softer EPON (A plus B 50–50) was used for red cells which had been aged or treated. Sections were viewed without staining, but lack of contrast made

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focusing difficult. Sections were stained with salts of lead and uranyl acetate and potassium permanganate (12). The sections were covered with carbon layer to prevent beam damage. RCA-2 and RCA 3E electron microscopes were used.

Reduced glutathione was measured according to the method for Gruenert and Phillips (13) as modified by Beutler (14).

For incubation studies, venous blood was collected with oxalate and when blood was collected from the finger tip, 0.01 ml was placed in test tube with 0.1 ml of an oxalated saline solution. Some samples were collected in heparinized capillary tubes. Glucose saline solution used in some experiments was prepared by adding 2.5 mg of glucose to 10 ml of saline.

The experiments were carried out in the blood of 8 normal adults, 40 prematures, and 40 full-term normal infants. A case of methylene blue intoxication is also described.

Results

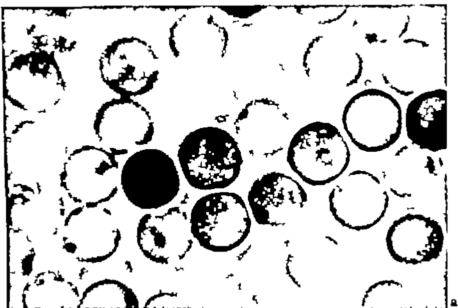
When samples containing acetylphenylhydrazine were constantly aerated in a Dubnoff shaker at 37 C and compared with an unaerated control increased rate and degree of Heinz body formation was seen. A decline in reduced glutathione correlated with these changes. After 60 min incubation a sample examined by electron microscopy showed inclusions singly and in clusters concentrated along the cell margin. Samples similarly treated at room temperature showed no Heinz body formation Likewise, under nitrogen no Heinz bodies were seen. Blood of prematures showed a much greater susceptibility to Heinz body formation than adult blood

The spontaneous formation of Heinz bodies was also studied. At 8 C there was no evidence of Heinz body formation for 7 days. At 37 C these inclusions were first seen at 48 h. With aeration Heinz bodies were seen at 24 h. After 72 h aeration electron microscopy revealed many changes (Fig 1)

Observation of blood diluted in saline and aerated at 37 C revealed increased Heinz body formation in premature's blood compared to that of adults (Table I) Electron microscopy of a premature's blood incubated for 72 h revealed changes similar to those noted in the blood of an adult, but more extreme (Fig 2)

Dilution of blood accelerated Heinz body formation (Table II) Electron microscopy of an 80 min sample diluted 1 10 showed slight irregularity of the cell membrane with the formation of

Fig 1 ( ) Microscopic ppearance of normal blood after aeration in the shaker for 24 h. Small single inclusions the margin of the cells (1000) (b) Electron micrograph of normal blood incubated for 72 h. Inclusions along the margin and similar bodies in the surrounding medium (18000).



a

0 50μ

b

small dense membrane-enclosed inclusion bodies usually near the margin.

The rate of Heinz body formation in blood of adults and prematures was then compared at 24 C 0 01 ml of blood was diluted in 0.2 ml of saline. Heinz bodies appeared at a more rapid rate in the blood of the prematures, and they tended to be more numerous and to be larger than those in adult blood (Table III)

Table I

Comparison of rate of spontaneous Heinz body formation in the blood of premature and of an adult.

Time	Premature % of cells with Heinz bodies	Premature Description	Adult % of cells with Heinz bodies	Adult Description
15 min	7	very small (one each cell)	0	
4 h	17	very small	0	
24 h	100	many in each cell	100	many in each cell

Table II

Effect of dilution of blood on degree of Heinz body formation aerated in shaker and incubated in water bath. Experiments were at 37 C. The numbers refer to percentage of cells containing Heinz bodies.

	Shaker				Water bath			
Time min.	undiluted	1 10	1 100	1 500	undiluted	1 10	1 100	1 500
40	0	40	50	70	0	13	17	20
100		—	—	—	0	15	70	80
120	0	80	100	100	0	50	80	90

Table III

The rate of Heinz body formation in blood from normal premature and from an adult. Experiments were carried out at room temperature. Numbers refer to percentage of red cells containing Heinz bodies.

Time min.	Premat. 0.01 blood and 0.2 oxalated saline	Premat. 0.01 blood and 0.2 oxalated saline and glucose	Adult 0.01 blood and 0.2 oxalated saline	Adult 0.01 blood and 0.2 oxalated saline and glucose
10	45	43	0	0
15	50	45	0	0
40	70	56	3	2
60	100	80	9	7
90	100	100	11	9
120	100	100	19	17
240	100	100	29	27

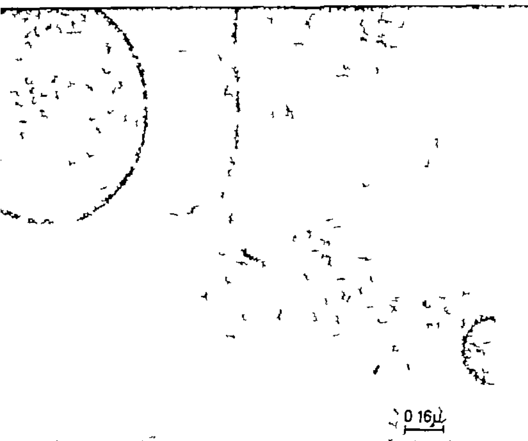


Fig 2. Electron micrographs of premature blood incubated for 72 h (18000) An apparent budding process of membrane and membrane-enclosed bodies within and without the cell.

Samples of blood from 40 premature infants with no abnormal clinical findings were examined for Heinz bodies. The age of these prematures varied from 1 to 25 days and the weights from 2 to 5 6 pounds 0 01 ml collected in 0.1 ml of saline was examined within 20 min. In 32 of these, 20 / of the red cells had single, small, refractile and usually peripheral inclusions. There was no correlation between their size and the age or birthweight of the premature Of 40 normal full-term infants 17 had small, single Heinz bodies in 2 / of the red cells. They equalled the smallest seen in the prematures. The remaining showed none. Control adult samples were negative

The blood of a case of anemia induced by methylene blue was also examined. Electron microscopy revealed striking changes, with marked fragmentation of the red-cell membrane and heavily stained conglomerate masses throughout the preparation (Fig 3)

Discussion

While the nature of Heinz bodies is not completely clear investigations by Beaven and White (15) Harley and Mauer (16) and Jandl *et al* (17) give strong evidence that they may be precipitates resulting from oxidative degradation of hemoglobin. Such a concept is consistent with the acceleration of their development *in vitro* in the presence of acetylphenylhydrazine, which acts as an oxidant, and inhibition of their development under a nitrogen atmosphere. It explains their presence in the early stages of drug induced hemolysis which was strikingly demonstrated in the case of methylene blue intoxication. Their spontaneous appearance *in vitro* was also accelerated by aeration and was retarded by incubation in the cold.

Under similar circumstances, it seems evident that the rate of formation differs in the blood of infants and in the blood of adults. The sensitivity of the erythrocytes in full-term infants is less than that of the smaller infants but more than that of adults. Whether fetal hemoglobin is more susceptible to oxidation is not known. There is a relative deficiency of methemoglobin reduction (18, 19) which could contribute to the increased susceptibilities in the newborn period

Variations in the appearance of the inclusions seem mainly to be a function of size. They may be evident as dust like particles along the margin of the cells, such as those which appear spontaneously on aeration of erythrocytes *in vitro* they may be seen as numerous small granules throughout the cell, or they may appear as clusters as large as $^1\!/_3$ of the cell diameter It is possible that all these are stages in the development of Heinz bodies, and it is clear that the rate of formation seems to control the size.

From electron microscopy one has the impression that these small masses arise at or near the cell membrane, and are often,

Fig 3. Blood sample from patient with methylene blue intoxication. ( ) Light microscopy shows large Heinz bodies in all cells (× 1000). (b) Electron micrograph demonstrates extreme fragility of membrane and heavily stained aggregates (× 18000)



0 53μ

b

themselves, enclosed by a membrane. They may form by budding or may actually be extruded by the cell, a phenomenon also noted on light microscopy. The dense masses which are without a definite membrane and are seen both within the cell and floating free, may either be a later stage or a different type of inclusion. High magnification of these demonstrates a definite structural pattern, which could be consistent with a change in configuration of the hemoglobin molecule. The differences in the appearance of the red cells of the adult and the premature on incubation appear to be more a matter of degree than of kind.

Changes in the membrane are of special interest since they may be more critical in the eventual hemolytic event of drug toxicity or of simple aging than the presence of Heinz bodies within the cell. These structural changes are consistent with biochemical changes which have been described in erythrocytes incubated with acetylphenylhydrazine *(20)* and may reflect chemical damage to sites susceptible to oxidation. That the red-cell membrane of the premature is also more susceptible than that of the adult cannot be determined from the present experiment. It is true, however, that the electron microscopic preparations of the former showed more distortion of cell outlines and more empty cells than those of the latter. Although many of the findings of electron microscopy may be artifacts of preparation and fixation and may, therefore, not be a direct reflection of the *in vivo* structure of the cells, the changes demonstrate a susceptibility to damage not seen in normal cells.

The findings are consistent with clinical observations suggesting increased susceptibility of the premature and normal newborn infant to hemolytic anemias characterized by Heinz bodies.

Summary

Heinz body formation has been investigated and compared in the blood of prematures, full-term infants and adults. Factors which can be demonstrated to increase rate of Heinz body formation include constant aeration at 37 °C and dilution of the sample, while nitrogen atmosphere and cold inhibit it. Electron microscope observations demonstrate changes in cell contents and in membrane when Heinz bodies are formed. Comparison with the blood of adults reveals that the blood of newborns is more susceptible to Heinz body formation with that of prematures being most susceptible.

Zusammenfassung

Die Bildung von Heinzschen Innenkörpern wurde untersucht und verglichen im Blut von Frühgeburten, von am Termin geborenen Kindern und von Erwachsenen. Die Bildung von Innenkörpern wird gesteigert durch konstante Belüftung bei 37 °C

und durch Verdünnung der Blutproben, sie wird gehemmt durch eine Stickstoffatmosphäre. Elektronenmikroskopische Untersuchungen ergeben Veränderungen des Zellinhaltes und der Membran, wenn Innenkörper gebildet werden. Der Vergleich mit dem Blut von Erwachsenen ergibt, daß das Blut von Neugeborenen gegenüber der Innenkörperbildung empfindlicher ist, wobei dasjenige von Frühgeburten am empfindlichsten ist.

Résumé

La formation d'inclusions de Heinz a été étudiée et comparée dans le sang de nouveau-nés prématurés, de nouveau-nés venus à terme et d'adultes. La formation d'inclusions est augmentée par une aération constante à 37 °C et par la dilution des prélèvements de sang; elle est inhibée par une atmosphère d'azote et par le froid. L'observation au microscope électronique met en évidence, quand des inclusions sont formées, des changements du contenu et de la membrane cellulaire. La comparaison avec du sang d'adultes montre que le sang de nouveau-nés est plus susceptible de former des inclusions de Heinz, le sang de nouveau-nés prématurés l'étant le plus.

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Knochenmarksveränderungen bei Typhus abdominalis*

R. J. Oechslin

In den letzten Jahren ist der Typhus abdominalis in Mitteleuropa durch die vielen Gastarbeiter wieder häufiger aufgetreten. Bei jedem unklaren Status febrilis muß deshalb auch immer die Möglichkeit eines solchen Infektes erwogen werden. Nachstehend möchten wir anhand von 7 Fällen über charakteristische Veränderungen des Blutes und des Knochenmarkes berichten.

Peripheres Blutbild

Wichtige Hinweise für die Diagnosestellung geben die Veränderungen des peripheren Blutbildes, welche in klassischer Weise von Naegeli (1) beschrieben wurden. Bei allen unsern 10 Fällen der letzten 8 Jahre konnten wir ebenfalls einen gleichartigen Ablauf erkennen. Zu Beginn, d.h. während der ersten Fieberperiode, trat ein Abfall der Leukozyten ein mit ausgeprägter Neutrophilie und Linksverschiebung. Die Leukozyten zeigten dabei ***ausgeprägte toxische Veränderungen***, die sich in Vergrößerung der Granulationen, Reifungsstörungen und Vakuolisierung des Protoplasmas und Verklumpung des Chromatingerüstes der Kerne äußerten. Bekannt ist vor allem das Fehlen der eosinophilen Leukozyten in den Anfangsstadien, sofern kein Asthma bronchiale oder andere allergische Erscheinungen vorliegen (Ekzem usw.).

Die Lymphozyten waren absolut vermindert, stiegen dann im zweiten Stadium deutlich an. Es kam deshalb häufig zu der bekannten Kreuzung der Lymphozyten-Kurve mit derjenigen der Neutrophilen. Der Thrombozyten-Abfall ging in der Regel der Schwere der Erkrankung parallel. Die tiefsten Werte von 40000 beobachteten wir bei einem sehr toxischen Falle, der sich initial während

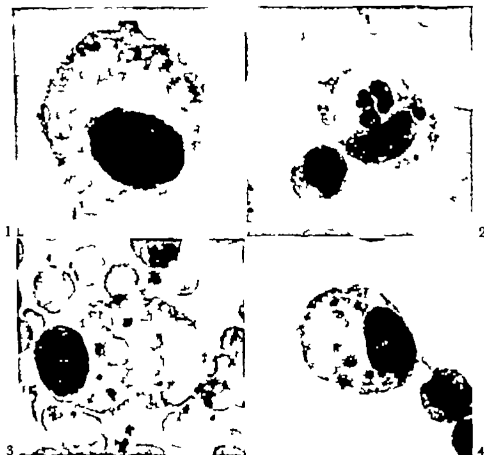
* Arbeit ausgeführt mit Unterstützung des Schweizerischen Nationalfonds zur Förderung der wissenschaftlichen Forschung Nr. 3587

mehrerer Tage im Delir befand. Der weitere Ablauf der peripheren Blutveränderung wurde durch die heutige wirksame Antibiotikatherapie wesentlich verkürzt. Meist kommt es innerhalb von zwei Wochen zur Normalisierung und Rückbildung der toxischen Veränderungen.

Knochenmarksveränderungen

Bei unseren 7 Patienten fanden wir sehr einheitliche Veränderungen des Knochenmarks. Die Zusammensetzung des Sternalmarkes beim Typhus abdominalis (2, 3) ist bisher vor allem durch SHIMOYAMA (4) beschrieben worden. Er nahm bei 11 Fällen in 5- bis 7tägigen Intervallen Sternalmarkuntersuchungen vor, ohne daß er aber auf die von uns beobachteten Zellmakrophagen und Granulome hinwies.

Bei unseren Patienten führten wir jeweils am 6. bis 7. Tag nach Beginn der Fieberschübe und in der Rekonvaleszenz eine Sternalpunktion durch. Zu Beginn waren die Ausstriche sehr zellreich. Die gesteigerte Myelopoese beherrschte das Bild und zeigte eine ausgesprochene Linksverschiebung. Die selektiv gesteigerte Granulozytopoese zusammen mit der peripheren Leukopenie spricht dafür, daß es sich bei der peripheren Leukopenie um einen durch die *Splenomegalie* verursachten vermehrten Abbau in der Milz und möglicherweise auch durch den infolge des schweren Infektes bedingten Mehrverbrauch der Granulozyten in der Peripherie handelt. In diesem Sinne spricht auch die ausgesprochene Linksverschiebung der Myelopoese im Mark, wie wir sie auch bei andern Leiden mit peripher gesteigertem Leukozytenabbau gefunden haben (5). Wie im peripheren Blutbild fanden sich auch im Knochenmark ausgeprägte toxische Veränderungen. In allen Ausstrichen waren eosinophile Myelozyten und Leukozyten nachweisbar, die zu diesem Zeitpunkt in der Peripherie vollständig fehlten. Die Erythropoese trat quantitativ deutlich zurück, wobei ebenfalls eine Linksverschiebung mit Vorherrschen der unreiferen Formen erkennbar war. In der Regel waren die *plasmazellulären Retikulumzellen* deutlich vermehrt, in einzelnen Fällen auch die kleinen lymphoiden Retikulumzellen. Es ließen sich auch reichlich Megakaryozyten nachweisen, z. T. mit Tendenz zu unreifen Formen. Diese Veränderungen klangen innerhalb von 2 bis 3 Wochen ab, und das Sternalmark zeigte dann praktisch ein unauffälliges Bild.



Abbildungen. 1 Große Retikulumzelle mit alveolär entmischtem Protoplasma. *2–4* Große Retikulumzellen mit phagozytierten Erythrozyten *(3 4)* neutrophilen Leukozyten *(2)* und Thrombozyten *(4)*

Als auffälligster Befund fanden sich in unseren 7 Fällen eigenartige *Retikulumzellen welche eine ausgeprägte Phagozytose zeigten* (Abb 1 bis 4) Sie waren meist locker in den Ausstrichen eingestreut, seltener herdförmig angeordnet. Es handelte sich dabei um relativ große Zellen mit breiten, scharf begrenzten Protoplasmasäumen. Das Protoplasma selbst war meist hell getönt, z. T fein vakuolar entmischt. Als Ausdruck einer regen Phagozytose fanden sich meist Erythrozyten pyknotische Leukozytenkerne und häufig auch Thrombozyten eingeschlossen. Die Kerne waren relativ klein, meist

längsoval, selten gelappt und in der Regel randständig, die Chromatinstruktur feinretikulär, seltener dichter zusammengesintert.

Zweifellos müssen diese Zellen den Makrophagen zugerechnet werden. Durch die eingehenden histologischen Untersuchungen ist ja bekannt (6, 7), daß der Typhus neben den typischen Darmveränderungen zu Knötchenbildungen in Leber, Milz und Knochenmark und seltener in den Lymphknoten führen kann. Es werden dabei im wesentlichen zwei Typen unterschieden. Der eine Typus entspricht einer umschriebenen Nekrose mit zellulärer, vor allem retikulärer Reaktion. Anderseits können sich eigentliche Knötchen, sogenannte Typhome, entwickeln, die einer Anhäufung protoplasmareicher retikulärer Zellen entsprechen. Zum Teil können sie mehr epitheloidzellartigen Charakter annehmen. Sie zeigen meist eine ausgeprägte Phagozytose. *Die Annahme liegt nun sehr nahe, daß es sich bei den in unsern Sternalmarkausstrichen beobachteten Zellen um solche «Typhuszellen» handelt, da wir sie vorwiegend bei Typhusfällen und viel seltener bei andern septischen Erkrankungen nachweisen konnten.*

Durch das Ausstreichen des Knochenmarkes auf die Objektträger würden diese Knötchen auseinandergerissen und damit eine lockere Durchsetzung des Knochenmarkes mit Typhuszellen vortäuschen.

Der Nachweis zahlreicher solcher retikulärer Zellen mit ausgeprägter Phagozytose hat eine große praktische Bedeutung für die Frühdiagnose des Typhus abdominalis. Das Sternalmark kann uns eine wesentliche Stütze bilden. Gleichzeitig möchten wir darauf hinweisen, daß der Erregernachweis im Sternalmark viel leichter gelingt als im peripheren Blut. Es sollte daher neben den Ausstrichen immer mit der andern Hälfte des Punktats auch eine Kultur auf den entsprechenden Nährböden angelegt werden. Die Typhusbazillen sind bis zu 20 bis 30 Tage nach Beginn der Erkrankung nachweisbar (8). Das Sternalmark dient uns somit in zweifacher Weise zur Diagnosestellung eines Typhus abdominalis.

Zusammenfassung

Bei 7 Patienten mit Typhus abdominalis wurde das Knochenmark untersucht. Zu Beginn der Erkrankung zeigten die Markausstriche ein einheitliches Bild mit hyperplastischer und nach links verschobener Leukopoese. Wie im peripheren Blutbild fanden sich ausgeprägte toxische Veränderungen. Die Erythropoese hingegen war qualitativ vermindert mit Vorherrschen der mehr unreifen Formen. Als auffälligster Befund ließen sich diffus im Knochenmark eingestreut relativ große Retikulumzellen mit ausgeprägter Phagozytose nachweisen. Es handelt sich dabei sehr wahrscheinlich um eigentliche «Typhuszellen», wie sie in histologischen Untersuchungen bereits bekannt sind.

Summary

Bone marrow studies in 7 patients with acute typhoid fever revealed hyperplastic and left shifted granulocytopoiesis. This finding was considered to correspond with the pronounced toxic neutrophilic reaction in the peripheral blood observed. Erythropoiesis was decreased in the marrow, immature forms predominating. The most striking and unusual finding were diffusely scattered large reticular cells with characteristic features of phagocytosis. These phagocytic marrow cells apparently correspond to proper typhoid cells as they may be recognized in microscopic section materials.

Résumé

La moelle osseuse de 7 malades souffrant de fièvre typhoïde a été examinée. Au début de la maladie, les frottis montrèrent une leucopoïèse hyperplastique et immature. Des altérations toxiques très prononcées furent constatées, de même dans le sang périphérique. L'érythropoïèse était qualitativement diminuée, les formes immatures prédominant. Le changement le plus marquant était la présence de grandes cellules réticulaires montrant une activité phagocytaire très intense. Ces cellules correspondent très probablement aux cellules typhoïdes, bien connues de par les examens histologiques.

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Cell Proliferation in Leukemia during Relapse and Remission

II DNA Synthesis of Leukemic Cells in the Peripheral Blood in vitro

J. R. Schmid, R. J. Oechslin, P. G. Frick and S. Moeschlin

While in a previous report *in vitro* DNA synthesis of leukemic cells has been measured in the bone marrow the present investigation is directed to study the leukemic DNA index in the peripheral blood. Serial incubations of leucocytic blood suspensions with H^3 thymidine may reveal changes of the DNA index occurring in the course of the disease. The greatest interest in such a study is indeed, to recognize any changes in the DNA index that may ensue prior to clinical remission or relapse. A shift in the labeling index of leukemic cells is most likely to occur in such transitional phases of the disease. With this purpose in mind, 16 cases of different types of leukemia have been studied with H^3 thymidine at regular intervals by using an autoradiographic *in vitro* technique.

Methods of Study

The leucocytic buffy coat layer of peripheral blood was incubated by constant gentle shaking with 1 ml of serum in a siliconized container at 37 °C for a 1-hour-period. H^3 thymidine (spect. act. of 3.5 C/mM) was added to the incubation suspension at a concentration of 0.8 µc/ml. The sample was then smeared on gelatine coated slides, covered with Kodak AR 10 stripping film, developed with Kodak D-19 after 5 and 10 days of storage at 18 °C and stained with Giemsa at pH 6.5 according to a slightly modified method (30) of Pelc (26).

Most of the autoradiograms were evaluated by three independent examiners. If the number of non classifiable cells or lysed cells exceeded 10%, the run was discarded. In addition, the calculated DNA values of 5 and 10 days film exposure were compared and only those with close agreement were used. Results were based on counting 500 peripheral leukemic cells in 6 cases and 200 cells in 10 cases.

Patients

Sixteen leukemic patients (10 ♂ and 6 ♀) from the Departments of Medicine of Zurich and Solothurn, 25 to 74 years of age, were selected for the present study There were 4 cases of acute stem cell leukemia, 4 cases of acute myelogenous leukemia, 3 cases of acute monomyeloblastic Naegeli type leukemia, 3 cases of chronic myelogenous leukemia, and 2 cases of malignant reticulosis with leukemic transition.

DNA synthesis index *in vitro* was determined in the peripheral leukemic cells serially at regular intervals in these 16 patients. During the period of study 2 patients (cases 1 and 2) developed complete remission of blood and marrow while only partial, temporary remission occurred in 3 other instances (cases 3 to 5). In 11 cases the course of the disease was steadily progressive. In 6 of them (cases 6 to 11), the course was rather slow extending over total period from 3 to 8½ months, and in 5 of them (cases 12 to 16) it was acute, leading to the patient death already within 11 days in one instance and within less than 2½ months after the appearance of the first clinical symptoms in the others.

Results

Case 1 (N M. ♂ 1940) chronic myelogenous leukemia Treatment with Myleran induced a complete remission within 4 months leucocytes fell from 260000 to 10000 splenomegaly regressed and blood and marrow reverted to normal. The DNA index of the peripheral leukemic cells was already rather high with 8.5 % prior to therapy and increased further to a level of 11.5 to 15 0 % prior to the remission phase.

Case 2 (v A. K. ♀, 1914) chronic myelogenous leukemia Treatment with Myleran resulted gradually in a complete remission within one month (Fig 1) The drop of leukemic cells in the blood was associated with an increase of the DNA index from 9.5 to 17.5 %.

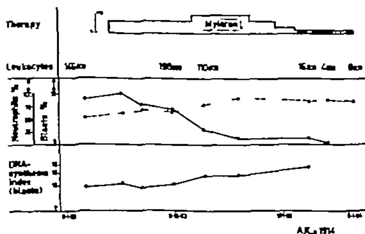


Fig. 1

Case 3 (K. J ♂ 1899) acute stem cell leukemia In this patient a partial remission lasting for 2 months could be achieved by the combined therapy with 6-Mercaptopurine and Millicorten (Fig 2) The percentage of blast cells decreased from 82 to 31 % At this time, the DNA index of leukemic cells changed from 3 6 to 9 4 %, this increase preceding the drop of peripheral blast cells by approximately 2 weeks. In a subsequent fatal relapse blast cells again prevailed in the blood with 90.5% and their DNA index decreased again to a level of 2.2 %

Case 4 (M. M. ♀ 1934) acute stem cell leukemia As seen in Fig 3 the course of the disease under therapy with 6-Mercaptopurine and Prednisone was biphasic, initiated by a gradual decrease of the white cell count and a drop in the percentage of blast cells. At this time the DNA index of 1 % prior to therapy changed to a level of 6 to 7 % Later on, another drop to 1 to 2 % preceded the terminal blastic crisis.

Case 5 (P H. ♂ 1936) chronic myelogenous leukemia As illustrated by Fig 4 continued administration of Myleran in high doses resulted after a 1 month period in a drop of the white cell count from 300000 to 20000 and of blast cells from 12 0 to 2.5 %. In the ensuing 4 months there was no further amelioration of the blood values. Prior to this partial remission the DNA index varied between 8 5 and 10.5 % and a decrease followed later to a level of 4 0 to 5 0 %.

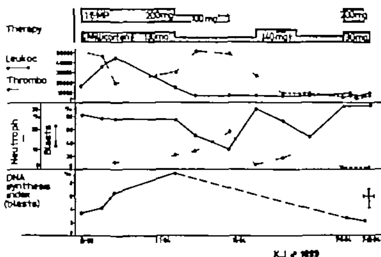


Fig 2

Fig 3

Case 6 (Z. E. ♀, 1902) acute stem cell leukemia. This patient was found to have a low-grade fever during a 4-months-period and an increase in immature forms was noted in the marrow before leukemic blast cells appeared in the peripheral blood. These cells were present in a fairly constant number of 21 to 33 %, the white count was constant at about 3000 and the differential count revealed a leukemic hiatus. Because of the pancytopenia therapy had

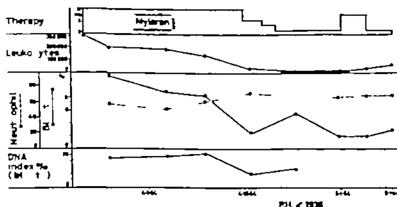


Fig 4

to be delayed. In the initial phase, DNA index of leukemic cells in the blood measured 7.5 to 10.5 %. Two months later an acute rise of blast cells to 81 % occurred and the patient succumbed.

Case 7 (S D ♀, 1901) acute myelogenous leukemia Treatment with Prednisone and later on 6-Mercaptopurine had no influence on the progressive course of the disease and the patient died within 3 months. Only during the terminal phase 33 % of leukemic cells appeared in the peripheral blood. The DNA index of these blast cells measured 7.2 to 8.0 %

Case 8 (B A. ♂ 1904) acute myelogenous leukemia This patient with chronic myelogenous leukemia adequately controlled over 5 years developed an acute phase with increasing size of the spleen, a leucocyte count of 63 250 with a fairly constant number of about 8 to 12 % blast cells in the blood. In spite of Cyclophosphamide, administered in a dose of 150 to 200 mg daily the clinical course was progressive, leading to death after 5 months. The DNA index of these peripheral leukemic blast cells was 10 to 11 % initially increased to 13.5 to 14.5 % immediately after the institution of therapy and decreased to a level of 4.5 to 6.5 % prior to the patient's death.

Case 9 (H M. ♂ 1914) leukemic phase of malignant reticulosis As seen from Fig 5 therapeutic attempts with Methylhydrazine, Velbe and Prednisone were of no avail on the course of the disease. Leukemic cells constituted between 60 and 80 % of peripheral white cells and the patient succumbed 3½ months after the institution of therapy The DNA index of blast cells was 7 to 8 % in the initial phase and only about 4 % terminally

Case 10 (H. B. ♀, 1897) acute monomyeloblastic leukemia The course of the disease was slowly progressive in this patient, leukemic cells representing consistently 70 to 80 % of nucleated blood elements (Fig 6) In spite of therapy with 6-Mercaptopurine and Prednisone, evidence of remission could never be achieved and the patient succumbed 8½ months after the onset of symptoms. The DNA index of the peripheral leukemic cells remained low within a range of 0.5 to 2.0 %.

Case 11 (W E. ♀, 1891) acute monomyeloblastic leukemia During the 8-months course of the disease (Fig 7) therapy with Prednisone and 6-Mercaptopurine resulted in some evidence of improvement with a decrease of leukemic cells in the blood from 18 to about 2 to 4 % and an increase in platelets. The DNA index was

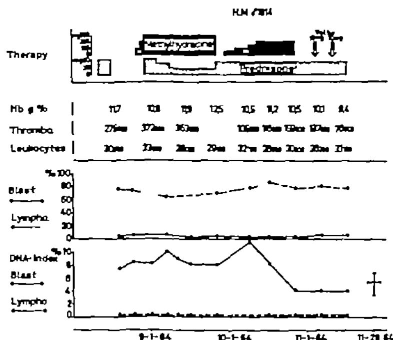
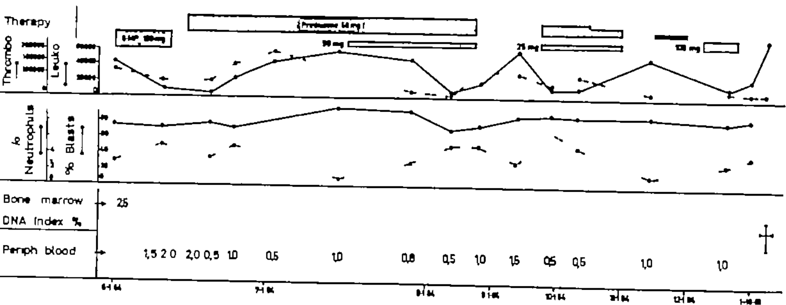


Fig 5

0.5 to 1 0 / initially and remained at about 2.0 / during this phase. Subsequently there was a drop in neutrophils to almost zero and blast cells increased to approximately 20 / before the patient succumbed after 6 weeks. In this terminal phase DNA values fell to 0.5 /.

Case 12 (S A. ♀, 1896) acute monomyeloblastic leukemia As shown in Fig 8 the course of the disease was acute and progressive in this patient in spite of therapy with 6-Mercaptopurine and Prednisone, leading to death within 2 months. The DNA index remained consistently low during this time, being 1 0 to 2 0 / in myelocytic blast cells and 0.5 to 4 0 / in monocytic blast cells.

Case 13 (H W ♂ 1906) leukemic phase of malignant reticulosis Fig 9 demonstrates that the disease was aleukemic during the early phase. Combined therapy with Endoxane and Prednisone proved to be of no avail. One month prior to death a sudden influx of leukemic cells occurred into the blood. In this terminal phase more than 80 / of nucleated forms were blast cells in the blood, and their DNA index varied between 4 0 to 5.5 /.



Therapy
Thrombo
Leuko
% Neutrophils
% Blasts
Bone marrow
DNA Index %
Periph blood
2,5
1,5 2 0
2,0 0,5
1,0
0,5
1,0
0,8
0,5
1,0
1,5
0,5
0,5
1,0
1,0
50 mg
25 mg
H.B. ♀ 1897

Fig 6

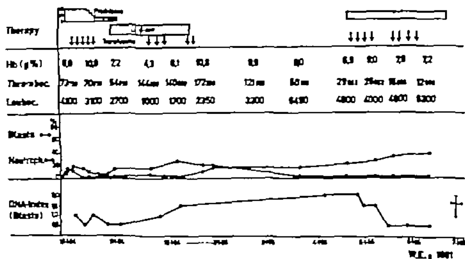


Fig 7

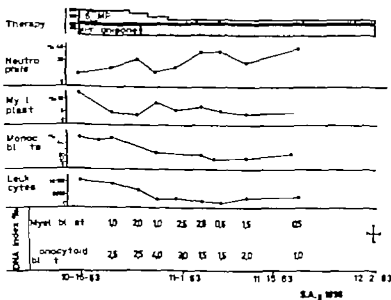


Fig 8

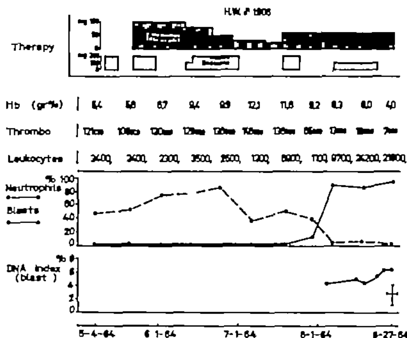


Fig 9

Case 14 (W O ♂ 1937) acute stem cell leukemia In spite of high doses of Millicorten (120 mg daily) combined with 6-Mercaptopurine (200 mg daily) the course of the disease was highly acute resulting in death within 11 days. Leucocytes increased from 3000 to 42000 in spite of this intensive therapy The number of blast cells varied between 75 and 83 %, and the DNA index during the acute terminal phase was 3 4 %.

Case 15 (W G ♂ 1902) acute myelogenous leukemia After a 6 year's chronic course, the acute leukemic phase terminated within 7 months, 6-Mercaptopurine being of no influence. Few days before the patient succumbed, leucocytes numbered 461000 with 79 % blast cells in the blood. H^3 thymidine labeled only 0.4 to 2 0 % of these peripheral leukemic cells.

Case 16 (S J ♂ 1891) acute myelogenous leukemia After a 6-months-period of pancytopenia a gradual increase in the number of blast cells was first noted in the bone marrow amounting to 31 % before an influx of these immature cells occurred into the peripheral blood. In the final 5 weeks there were approximately 9 % blast cells in the blood and the level of labeling with H^3 thymidine was quite low ranging from 0.5 to 1 0 %.

Discussion

The 1 hour DNA synthesis index of peripheral leukemic blast cells was evaluated in the present study by an autoradiographic *in vitro* technique. Results in 16 investigated cases revealed that in the acute phase leukemic cells were labeled in 0.4 to 10.0 (in a single case 14.5) % with H^3 thymidine. This DNA index is lower than in chronic myelogenous leukemia where the index ranged from 4.0 to 17.5 %. The lowest and most consistent values in the course of the disease ranging from 0.5 to 3.5 % in 35 determinations, were computed in 3 cases of monomyeloblastic Naegeli type leukemia. A change in the DNA index of peripheral leukemic cells could be recognized only prior to a remission phase or while transition to a relapse took place.

The observation of low DNA values in leukemia has been made previously by several investigators (1 3 5 10 14 15 18, 19 21). These findings clearly differ from those obtained in proliferating immature cells of the normal bone marrow. In one study (30) the labeling index with H^3 thymidine was found to be 35 % for myelocytes, 61 % for basophilic normoblasts and 23 % for polychromatophilic normoblasts. In the entire series of 16 cases described here, the DNA level of peripheral leukemic cells ranged only from 0.4 to 17.5 %. Although a direct comparison between peripheral and bone marrow cells is not possible, it is interesting to observe such a low proliferative index in peripheral leukemic blast cells.

The low DNA values may be the expression for a prolonged life span of leukemic cells, an assumption for which considerable evidence has been accumulated today (4 11 12, 24 25 32). The use of an *in vitro* method alone, however would not contribute much to clarify this problem and subsequent *in vivo* studies are necessary in this regard. If the population of leukemic cells in the peripheral blood, although morphologically homogeneous, would be heterogeneous regarding cellular functions, their proliferative activity might differ also. A part of leukemic cells might belong to a rather slowly proliferating population and some of them may even be entirely outside of the generation cycle in the sense of end cells (4 6, 21–23). In addition, the DNA synthesis period itself could be shorter in leukemia which might cause the observed low values of *in vitro* DNA synthesis.

Results of the present study are indicative of controversial DNA values between acutely progressive leukemias and those just prior to a remission phase the computed values were markedly lower in the first group than in the latter (Table I and Fig 1–9) These data seem to be paradoxical since in the acute phase of the disease one would rather expect an increase of the DNA index of leukemic cells along with the rising number of blast cells in the peripheral blood.

The low DNA index during relapse might indicate that immature leukemic cells have lost the ability of maturation. The basic defect would then not be in cell production but rather in the elimination of leukemic cells. A disturbance of this kind would lead even in the presence of very slowly proliferating cells to the tremendous bulk of pathological cells in the blood and tissues, as noted in this disease. There is evidence to assume that peripheral leukemic cells may represent a conglomerate of several populations with different proliferative activities (10 21 22) In the problem of production and removal it may be conceivable that the population of blast cells may change during the course of the disease. The acute phase might be associated with the predominance of a more slowly proliferating population and remission may be induced by the appearance of a more actively proliferating form of immature cells (4 21 22) *In two* studies have not as yet been conclusive (5 9, 15 17 21) They are, in general, indicative of a prolonged generation time of blast cells (4 9 14 22) Studies of the total life span of leukemic cells of bone marrow and peripheral blood revealed generally prolonged values (12 24 25 32)

It is possible that the increasing DNA index noted prior to a remission phase may be the expression for the transition towards a more normal pattern of cell function. Remission could then be initiated whenever the immature cells regain properties for an accelerated cell cycle. Elimination of leukemic cells could subsequently occur over cellular maturation which is the fate of normal blood cells (9) At the time of early remission, a morphologically not distinguishable population of immature cells would be produced, differing from the immature cells of acute leukemia by the presence of an intact enzyme system enabling these cells to undergo a normal postmitotic maturation. Marsh *et al* (20) noted, indeed, higher values of thymidine phosphorylase in leukemic cells at the time of remission than during the acute crisis.

Such an interpretation of the data (Table I) would also be in keeping with the well known observation that during remission immature leukemic cells with a low DNA index gradually disappear while more mature forms which are known to have a higher DNA index begin to repopulate the bone marrow A non-specific, purely antimitotic effect of chemotherapeutic agents could certainly not explain these morphologic changes.

Remission could be induced by the removal or inactivation of one or several factor(s) that inhibit during the acute leukemic phase the normal development to more mature cell types. *In this sense inhibition of a viral factor has to be considered as being one possible explanation, analogous to the virus induced leukemias in which subsequent to the infection a disturbance of cellular maturation has been recognized* (2, 7 8, 13, 16, 27 32) Inhibition of viral multiplication has been demonstrated indeed for the most commonly used chemotherapeutic agents (28, 31 33)

The observed changes of the DNA index of leukemic cells during the evolution of the 16 cases described here are certainly remarkable. Comparable differences of the DNA index prior to remission and relapse have been recognized previously (29) in leukemic cells of the bone marrow of 22 patients. The labeling index of leukemic cells has in general been noted to be a little lower in the peripheral blood than in the marrow (14 22, 29) Therefore, the simultaneously obtained DNA values of leukemic cells in marrow and blood cannot be directly correlated. It is noteworthy however that there appears to exist a similar pattern of the DNA index in leukemic cells of the blood and marrow when transition from relapse to remission and vice versa occurs (29) Our observations are in keeping with the data of KILLMANN *et al* (15) who studied the clearance of labeled blast cells from the blood and found that the influx of labeled and unlabeled blast cells into the peripheral blood had a ratio that remained fairly constant.

It ought to be emphasized again that due to the existing possibility of a functionally non-uniform population of leukemic cells in blood and marrow only assumptions about the basic process leading from relapse to remission may be drawn from the *in vitro* studies described above. *In vivo* investigations, that are in progress, determining cycle times and DNA synthesis periods of leukemic cells prior to remission and during the acute phase, shall further contribute to elucidate this complex problem.

Table I

Summary of the DNA synthesis dat of the 10 cases studied with regard to their clinical course.

Prognostic group		Case No.	Type of leukemia	DNA synthesis index in per cent		
				prior to therapy	prior to remission	acute terminal phase
Cases with remission phase	complete	1	chronic myelogenous	0.5	11.5—15.0	
		2	chronic myelogenous	0.5	17.5	
	partial	3	acute stem cell	3.6	9.4	2.2
		4	acut stem cell	1.0	6.0—7.0	1.0—2.5
		5	chronic myelogenous		8.5—10.5	4.0—5.0
Cases without remission phase	steadily progressive	6	acute stem cell	7.5—10.5		5.0—7.0
		7	acute myelogenous			7.2—8.0
		8	acute myelogenous	10.0—14.5		4.5—0.5
		9	malignant reticulosis	7.0—8.0		4.0
		10	acute monomyeloblastic	1.5—2.0		0.5—1.0
		11	acute monomyeloblastic	0.5—2.0		0.5
	acute	12	acute monomyeloblastic	1.0—3.5		0.5—2.0
		13	malignant reticulosis			4.0—5.5
		14	acute stem cell			3.4
		15	acut myelogenous			0.4—2.0
		16	acute myelogenous	1.5—2.0		0.5—1.0

Acknowledgment. We are indebted to Miss E. Sem, Miss L. Koch and Mrs. U. Gerra for their excellent technical assistance in performing the autoradiographic studies.

Summary

Serial studies of the DNA synthesis index *in vitro* in peripheral blast cells revealed in 16 patients with leukemia fairly stable values only for the individual cases. However in the group of 3 chronic myelogenous forms values were higher (4.0 to 17.5%) than in 10 acute myelogenous and stem cell leukemias (0.4 to 10.0%). Lowest values were recorded in 3 monomyeloblastic types (0.5 to 3.5%). A consistent pattern in the change of this individual level of the DNA index was recognized only when transition from relapse to remission or vice versa occurred: the DNA index decreased prior to an acute phase and increased in the early remission phase. It appears likely from this study that acute relapsing leukemia is not primarily related to an accelerated cell proliferation. The increasing DNA index prior to remission may be indicative that removal of factors inhibiting the maturation process of immature cells may be more important to induce clinical remission than inhibition of cell proliferation itself.

Zusammenfassung

Serienuntersuchungen des Ein-Stunden-DNS-Synthese Index *in vitro* in leukämischen Zellen des peripheren Blutes ergaben relativ konstante Werte nur für die Einzelfälle. Dagegen waren die Werte bei 3 chronischen myeloischen Leukämien (4,0–17,5%) höher als bei 10 akuten myeloischen und Paramyeloblasten-Leukämien (0,4–10,0%). Die tiefsten Werte lagen bei 3 akuten Monozytenleukämien vor (0,5–3,5%). Ein grundsätzliches Prinzip im Wechsel dieses individuellen DNS-Spiegels konnte nur beim Übergang zu einer Remission oder zu einem Rezidiv erfaßt werden. der DNS-Index fiel dabei vor der akuten Phase ab und stieg bei beginnender Remission wieder an. Auf Grund dieser Befunde erscheint es gegeben, bei akuten Leukämien nicht eine Beschleunigung der Zellproliferation als pathogenetisch wesentlichsten Faktor anzunehmen. Der zunehmende DNS-Index vor einer Remission weist vielmehr darauf hin, daß offenbar die Entfernung von Faktoren, welche in der akuten Phase die Zellreifung hemmen, wichtiger ist für die Einleitung einer klinischen Remission als die Hemmung der Zellproliferation an sich.

Résumé

Une série d'analyses concernant l'index de synthèse de l'ADN dans les cellules leucémiques du sang périphérique *in vitro* a donné des valeurs assez constantes au cours de chacun des 16 cas étudiés. Dans les 3 cas de leucémie myéloïde chronique, les valeurs étaient plus élevées (4,0–17,5%) que dans les 10 cas de leucémie myéloïde aiguë et paramyéloblastique (0,4–10,0%). Les valeurs les plus basses ont été observées dans 3 cas de leucémie monocytaire (0,5–3,5%). En général, le changement de ce niveau individuel de l'ADN n'a été observé qu'au début d'une rémission ou au début d'une rechute. On constata que l'index de synthèse de l'ADN diminuait avant et pendant la phase aiguë et augmentait de nouveau au début d'une rémission. D'après ces résultats, il faudrait conclure que dans les leucémies aiguës, l'accélération de la prolifération cellulaire n'est pas le facteur pathogénique principal. L'augmentation de l'index de l'ADN au début d'une rémission indique probablement que la libération des facteurs, empêchant la maturation cellulaire pendant la phase aiguë, pourrait être plus important que l'inhibition de la prolifération cellulaire elle-même, pour favoriser une rémission clinique.

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The Transformation of Human Mononuclear Leukocytes in vitro

II Precursors of Large Mononuclear Cells on Coverslips

J O Lamvik

In a previous paper (8) the gradual transformation of the cell picture in leukocyte coverslip cultures was described. After 48—72 h culture time the coverslips contained mainly large mononuclear cells, which were considered transformed types of leukocytes from peripheral blood as no sign of cellular proliferation was apparent in the cultures. The polymorphonuclear granulocytes showed signs of degeneration and it was presumed that the precursors of the large mononuclear cells were the small lymphocytes or the monocytes from the blood.

Two other possibilities, however have to be considered. Firstly band types of granulocytes, as they stick to the glass, might possibly be able to transform to large mononuclear cells. Secondly the large mononuclear cells could be degenerated forms of any or some of the cells that attach themselves to the coverslips.

The purpose of this investigation was to study these four possibilities. Pure lymphocyte cultures were compared with cultures containing all types of leukocytes. Secondly the influence of band granulocytes on the coverslip cell population was studied by comparing the leukocyte cultures from a patient with septicaemia with normal leukocyte cultures. Thirdly the cell vitality was evaluated from the nuclear fluorescence after Acridine Orange supravital staining Cells attached to coverslips as well as unattached cells in the culture medium were studied.

Material and Methods

Human blood was obtained by venepuncture and erythrocytes and thrombocytes separated as previously described (8). Parts of the leukocyte suspension were used in culture tubes with medium consisting of 70% Parker' tissue culture medium (TC 199) and 30% human plasma. Other parts were transferred to cotton wool columns for separation of the lymphocytes, according to method previously described (9). The lymphocyte suspensions were used in other series of culture tubes with medium as for the leukocyte cultures.

Coverslips were placed in all tubes and harvested in duplicate at regular intervals. In the first and second experiments the coverslips were fixed and stained with May Grünwald-Giemsa. In the third experiment the coverslips after dipping in saline to wash away non-adherent cells, were transferred to staining solution containing 4.5 mg Acridine Orange per 100 ml TC 199 pH 7.4 After 10 staining time the back of the coverslip was dried with cloth, the coverslip inverted on clean microscope slide and observed in ultraviolet light using Zeiss photomicroscope with BG 12 exciter filter and yellow-orange absorption filter

The cell numbers and differential counts on the coverslips in 4 high power fields, each having diameter of 0.4 mm, were estimated. In the Acridine Orange stained preparations the cells were also differentiated in vital and non-vital cells according to the nuclear fluorescence (6) This differentiation was completed within 1 to 2 min.

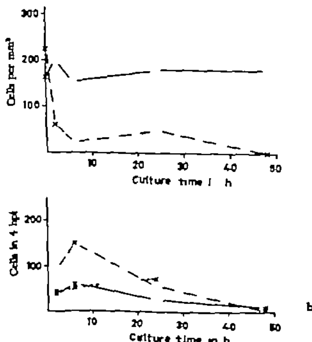


Fig 1 Variations in the numbers of unattached cells ( ) and cell numbers per 4 high power fields on coverslips (b) in leukocyte cultures. Each mark is the mean of the values from duplicate culture tubes. Granulocytes — ———— lymphocytes — ———, large mononuclear cells —

The non-adherent cells were mixed in the culture medium by rotation of the tubes and cell numbers determined, using standard haematological technique (2). In the first and second experiments differential counts of cells in suspension were estimated after preparation of smears stained with May-Grünwald-Giemsa. In the third experiment the cells were stained with Acridine Orange and examined in ultraviolet light (8) in order to estimate viability.

Results

First culture experiment Two series of culture tubes were prepared, one containing 1.5×10^6 leukocytes per tube in 3.5 ml medium, the other 1.5×10^6 lymphocytes per tube. Differential counts of the lymphocyte suspension before culturing showed 96% lymphocytes and 4% smudge cells.

The numbers and differential counts of unattached cells in suspension and cells attached to the coverslips are given in Tables I and II The variations in the numbers of the different types of unattached and attached cells are shown in Fig 1 and 2 The number of unattached lymphocytes was quite steady in leukocyte

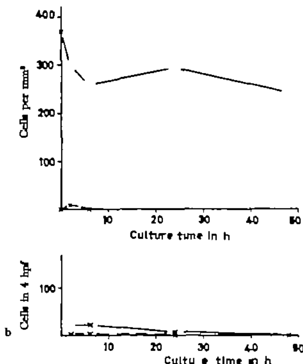


Fig 2. Variations in the numbers of unattached cells ( ) and cell numbers per 4 high power fields on coverslips (b) in lymphocyte cultures. Key as in Fig 1.

as well as lymphocyte cultures. In the leukocyte cultures large mononuclear cells were present on the coverslips after 2 h and dominated the cell picture after 48 h. In the lymphocyte cultures the cell number on the coverslips was significantly lower than in the leukocyte cultures, only a few small lymphocytes were found. In the late cultures the coverslips were almost free of cells. The number of damaged cells was quite high in the suspensions, while few cells with damaged nuclei were observed on the coverslips, probably because damaged cells had fallen off the glass surface.

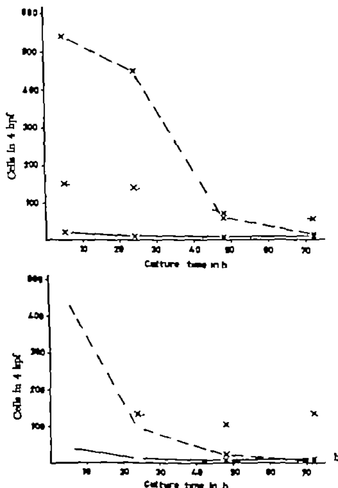


Fig 3. Variations in the numbers of cells per 4 high power fields on coverslips in leukocyte cultures from case of septicaemia with 7% juvenile granulocytes in the differential count ( ) and from normal blood donor (b) Key as in Fig 1.

Second culture experiment A series of culture tubes was prepared with leukocytes from a patient with septicaemia with 7% unsegmented granulocytes in the cell suspension added to the tubes. Another series of cultures was prepared from a donor with a normal differential count. Each tube in both series contained 3×10^4 leucocytes. The cell numbers and differential counts on the coverslips in the two series are given in Table III which also shows the differential counts in the leukocyte suspensions added to the culture tubes. Fig 3 shows the variations in the absolute numbers of cells in the two series. The results show that there is no significant difference between the numbers of large mononuclear cells on the coverslips in the two series.

Third culture experiment Two other series of culture tubes were prepared as in the first experiment. The vitality of cells on the cover slips as well as in the suspension was evaluated after Acridine Orange staining The cell numbers and differential counts and the variations in the numbers of cells with apple-green nuclear fluorescence in the leukocyte cultures are shown in Table IV and Fig 4 Almost all the

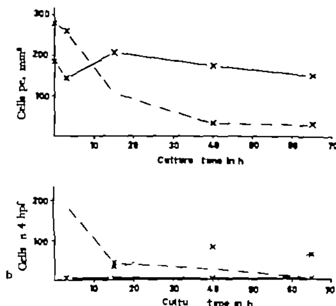


Fig 4 Variations in the numbers of vital unattached cells ( ) and vital cells per 4 high power fields on coverslips (b) in leukocyte cultures. Each mark is the mean of the values from duplicate culture tubes. Vital granulocytes = ——— vital lymphocytes = ——— vital large mononuclear cells =

Table I

Cell numbers and differential counts in leukocyte cultures (mean values from duplicate culture tubes).

	Culture time, h	Cell No.	Differential counts (%)			
			granulocytes	lymphocytes	large mononucl. cells	damaged cells
Unattached cells	0	390*	58	42	0	0
	2	325	19	62	0	19
	6	295	8	54	0	38
	24	305	16	59	3	22
	48	310	0	58	1	41
Attached cells	2	185	54	22	24	0
	6	270	55	23	21	1
	24	177	34	17	43	6
	48	90	7	11	82	0

Cell number per mm³ in suspension.
Cell number in 4 high power fields on coverslip.

Table II

Cell numbers and differential counts in lymphocyte cultures (mean values from duplicate culture tubes).

	Culture time, h	Cell No.	Differential counts			
			granulocytes	lymphocytes	large mononucl. cells	damaged cells
Unattached cells	0	385	0	96	0	4
	2	335	2	90	0	8
	6	345	0	76	0	24
	24	335	0	88	0	12
	48	330	0	75	0	25
Attached cells	2	24	2	22	0	0
	6	33	2	22	2	8
	24	10	0	8	2	1
	48	1	0	0	1	0

Cell number per mm³ in suspension.
Cell number in 4 high power fields on coverslip.
Percentage.
Absolute values.

large mononuclear cells on the coverslips in the leukocyte cultures showed a bright, apple-green fluorescence, a sign of undamaged nuclear chromatin (6) Among the unattached cells on the other hand, many cells showing red-orange or dull-green nuclear fluorescence were found probably due to cell degeneration or death. Most

Table III

Cell numbers on coverslips in leukocyte cultures from case of septicaemia (A) and normal blood donor (B) (mean values from duplicate culture tubes)

	Culture time, h	Cell No.	Differential counts (%) granulocytes juvenile	granulocytes mature	lymphocytes	large mononucl. cells	damaged cells
A. Before culturing			7	81	9	3	
Cells on coverslips	3	709		76	3	21	
	24	596		73	2	23	
	48	138		42	5	50	3
	72	80		15	11	70	4
B. Before culturing			1	42	50	3	
Cells on coverslips	5	615		73	7	20	
	24	244		40	5	55	
	48	141		15	6	75	4
	72	152		4	7	89	

Cell numbers in 4 high power fields on coverslips.

Table IV

Cell numbers, differential counts and vitality in leukocyte cultures (mean values from duplicate culture tubes)

Culture time, h	Cell No.	Differential counts (%) vital cells: granulocytes	vital cells: lymphocytes	vital cells: large mononucl. cells	non-vital cells: granulocytes	non-vital cells: lymphocytes	non-vital cells: large mononucl. cells
Unattached cells							
0	600*	47	31	0	22	0	0
3	545	48	27	0	23	2	0
15	593	18	35	0	45	2	0
40	455	7	39	2	45	7	0
65	510	7	30	1	56	6	0
Attached cells							
3	269*	71	2	15	12	0	0
15	121	37	4	32	25	2	0
60	135	20	3	60	11	3	3
65	82	0	2	70	22	3	2

Cell number per mm³ in suspension.
Cell number in 4 high power fields on coverslip.

of these cells were granulocytes. In the lymphocyte cultures 85—90% of the unattached cells showed green nuclear fluorescence throughout the culture time. Only a few lymphocytes were found on the coverslips, most of these were damaged.

Discussion

The large mononuclear cells were almost exclusively found in the leukocyte coverslip cultures. The lymphocytes in the effluents from the cotton wool columns showed a very slight tendency to stick to glass. Only about 1% of the lymphocytes added to the culture medium were found on the coverslips after 2 and 6 h culture time. These lymphocytes showed no tendency to transform to large mononuclear cells. The precursors of the large mononuclear cells thus appear to be cells that were retained in the cotton wool columns.

It is possible that some lymphocytes are more sticky than others and that these cells, being retained in the cotton wool columns, may act as precursors for the large mononuclear cells in the leukocyte cultures. The second culture experiment seems to rule out this possibility along with another possible precursor the unsegmented granulocyte. In this experiment the number of lymphocytes added to one of the culture series was 5 times as high as the number of cells added to the other series, without any difference being found in the numbers of large mononuclear cells. Furthermore, the unsegmented granulocytes being 7 times more numerous in one of the culture series, are unlikely to be precursors. The results gives good evidence that the monocytes are the precursors of the large mononuclear cells, as the monocytes are the only cells added to all the culture tubes in the same numbers. About 90000 monocytes were added to each tube. This number is high enough to account for all the mononuclear cells on the coverslips.

In the May-Grünwald-Giemsa stained coverslips the large mononuclear cells show sharply outlined nuclei, but the cytoplasm is often irregular and evaluation of cell vitality is impossible. The results of Acridine Orange vital staining show that the morphological changes which characterize the large mononuclear cells are not due to cellular degeneration. The irregularity of the cytoplasm is almost certainly due to its spreading on the glass. A higher proportion of cells with damaged nuclei were found in the suspension than on the coverslips. This is probably due to detachment of the cells when they degenerate.

The results obtained are consistant with the findings of Medawar (10) who observed no transformation of rabbit lymphocytes into macrophages in vitro, but correlated the number of phagocytes which appeared in the cultures with the number of

monocytes in the inocula. Similarly GOWANS (4) observed no transformation of rat lymphocytes in fluid chambers and using coverslips applied to areas of abraded skin in rats, in which different cells were labelled with tritiated thymidine (12) showed that the great majority of the small lymphocytes in the blood could be excluded as antecedants of the large mononuclear cells found on the cover slips. RABINOWITZ AND SCHREK (11) have reported studies on human leukocytes in slide chambers in which macrophages appeared to develop only from monocytes. On the other hand BERMAN AND STULBERG (1) culturing human leukocytes on coverslips considered lymphocytes to be the main source of the macrophages found They did not culture separated lymphocytes.

The present findings show definitely that the small lymphocytes in the effluents from the cotton wool columns do not transform to other cell types when cultured without stimulants. Furthermore, the cells do not adhere to glass. This render the cotton wool separated lymphocytes convenient cells for the study of the blastoid changes observed when antigens (3) or other stimulants (5 7) are added to the cultures.

Summary

A comparison of leukocyte and lymphocyte cultures has been performed and the numbers and differential counts of coverslip-attached and unattached cells determined. Large mononuclear cells were only found on coverslips in leukocyte cultures. The small lymphocytes showed slight tendency to stick to glass and did not transform to other cell types. Leukocytes with high proportion of band types of granulocytes gave no increase in the number of large mononuclears on the coverslips when compared with normal leukocyte coverslip cultures. The large mononuclear cells showed signs of nuclear vitality after Acridine Orange vital staining, and were obviously not derived from cells having undergone degenerative change in the suspension or on contact with the glass surface. The results are explained by the monocytes of the leukocyte suspensions being the main precursors of the coverslip mononuclear cells.

Zusammenfassung

Kulturen von Leukozyten und von Lymphozyten wurden verglichen. Dabei wurden die Gesamtzahlen und die Differenzierungswerte der an Deckglaschen haftenden und der freien Zellen bestimmt. Große mononukleare Zellen fanden sich nur an Deckgläschen in Leukozytenkulturen. Die kleinen Lymphozyten zeigten eine geringfügige Tendenz zur Haftung an Glas und wandelten sich nicht in andere Zelltypen um. Leukozyten mit einem großen Anteil an Stabkernigen ergaben keine Zunahme der großen Mononukleären an den Deckglaschen im Vergleich zu Deckglaskulturen normaler Leukozyten. Bei Vitalfarbung mit Akridinorange zeigten die großen mononukleären Zellen Zeichen der Vitalität ihrer Kerne sie leiteten sich keinesfalls von Zellen ab, die

in den Suspensionen oder beim Glaskontakt degenerative Veränderungen erlitten hatten. Diese Ergebnisse werden damit erklärt, daß die Monocyten der Leukocytensuspensionen die hauptsächlichen Vorstufen der mononukleären Zellen an den Deckgläschen sind.

Résumé

Des cultures de leucocytes et de lymphocytes ont été comparées. Leur nombre total et la différence entre le nombre des cellules fixées à des couvre-objets et celui des cellules libres ont été déterminées. Les grandes cellules mononucléaires ne se trouvèrent dans les cultures de leucocytes que sur les couvre-objets. Les petits lymphocytes ne montrèrent qu'une légère tendance à adhérer au verre et ne se transformèrent pas en d'autre types de cellules. Quand les leucocytes sont en grande partie non-segmentés, il ne se trouve sur les couvre-objets aucune augmentation des grandes cellules mononucléaires, à l'encontre des cultures contenant des leucocytes normaux. Les grandes cellules mononucléaires montrèrent des signes de vitalité nucléaire après coloration à l'orange d'acridine; elles ne dérivent apparemment pas des cellules qui ont subi des altérations dégénératives dans les suspensions ou au contact avec du verre. L'explication de ces résultats serait que les monocytes des suspensions de leucocytes sont les principaux prédécesseurs des cellules mononucléaires qui se trouvent sur les couvre-objets.

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Studies of Lymphocytes and Their Derivative Cells in vitro

II. Enzyme Cytochemistry

J Gough* and M W Elves

Lymphocytes are capable of transforming into macrophages *in vitro* (16) and when stimulated with phytohaemagglutinin (PHA) may develop into nucleolated pyroninophilic cells which have a morphological resemblance to haematological stem-cells (7 12, 22)

Certain biochemical constituents have been studied in these various cells (15) and in this study the activity of various enzymes will be compared in the lymphocyte, the macrophage, and the PHA transformed cell

Methods

Smears of leucocytes, macrophages and PHA-transformed cells were prepared as previously described (15)

Alkaline phosphatase. A modified technique was used (11), which is based on Kaplow's method (21).

Acid phosphatase The standard method of Grogg and Pearse (17) was used with smears fixed in formalin vapour for 5 min or unfixed.

Non-specific esterase The method was based on Gomori's modification of the technique of Nachlas and Seligman (26) which was applied to blood cell smears by Braunsteiner (4) using α-naphthyl acetate as substrate. Unfixed air-dried smears were treated at room temperature for 10 min in the substrate solution, fixed in Carnoy's solution, and counter stained. Control slides for phosphatases and esterases were treated in the same way but with substrate omitted from the medium.

5-Nucleotidase The method used was that described by Pearse (27) which was based on Wachstein (37) with the modification that unfixed air-dried smears were used, and the incubation medium was filtered before use additional lead nitrate solution was added until no further precipitate occurred, and the mixture refiltered. Control slides in which the substrate was omitted or the reaction inhibited with 10 mg of nickel sulphate were negative.

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* The contribution by this author formed part of an M. D. thesis accepted by the University of Cambridge.

Leucine amino-peptidase. The method was based on those of Burstone and Folk (6) and Nachlas *et al.* (25) using unfixed air dried smears, treated at 37 °C for 30 min in the incubation medium described by Pearse (27) with L-leucyl-β-naphthylamide as substrate. Slides were then washed, postfixed in 10% formol-saline and counterstained. The dye product will normally chelate with copper salts, but this was not found possible by the present authors, possibly due to the fact that the preparation of Fast Blue B (Sigma) contained zinc as stabiliser Control slides incubated in medium in which the cyanide was replaced by 0.1 M-copper-sulphate showed faint diffuse brown stain only

Dehydrogenases and diaphorases. Two problems were encountered in the demonstration of dehydrogenases. Firstly these enzymes are very vulnerable to unsuitable fixation and the present authors found unfixed smears preferable. Fixation in 60% acetone for 30 (28) is suitable, but there is some reduction in positivity Secondly control smears, in which substrate is omitted from the incubation medium, frequently show weak positive reaction ("nothing dehydrogenase") and it was thought that this might be due to the presence of various substrates or reduced coenzymes already in the cell. Test and control slides were therefore incubated for 3–10 min in isotonic saline before being treated with the main incubation medium in an attempt to remove such substances. In this way the nonspecific reaction was completely abolished, and the test reactions were only slightly affected.

The technique was otherwise that of Pearse (27) using the following substrates sodium DL-β-hydroxybutyrate, sodium L-glutamate, glucopyranose-6-phosphate disodium salt, 6-phosphogluconic acid barium salt, sodium DL-α-glycerophosphate, sodium DL-isocitrate, sodium -malate, sodium lactate, sodium succinate. Coenzyme best results were obtained when stock solution was made up freshly for each experiment. A solution of 0.1–0.2 M. TPN was used for pentose shunt enzymes, no coenzyme is required for succinic dehydrogenase, and DPN was used for the rest. 0.1 M-sodium-azide was used as respiratory inhibitor for pentose shunt enzymes, and 0.1 M-sodium-cyanide for the remainder

Saline treated, unfixed air-dried smears, not more than two days old, were incubated with the staining medium at 37 °C for 45 min (for glutamic, lactic, malic and β-hydroxybutyric dehydrogenases) and 90 min for the other enzymes. Slides were then fixed in 10% formol saline and counterstained with methyl green. Saline pretreatment was omitted for the demonstration of pentose phosphate shunt enzymes.

Diaphorases. Malic dehydrogenase was shown to be present in all the cells in the present investigation, and has the advantage that it may be used with DPN or TPN It may thus be used to demonstrate both diaphorases, using sodium malate as substrate, and omitting the coenzyme for controls. The method is the same as that for dehydrogenases, using DPN with one set of slides, and TPN with the other

Phosphorylase. The method was that of Takeuchi and Kinoshita (34) modified by Quaglino and Hayhoe (29) except that acetone fixation was omitted.

Results

Alkaline phosphatase In buffy coat smears about 20–40 / of polymorphs showed positivity in the form of discrete brown granules superimposed on a diffuse brown background stain lymphocytes and monocytes were negative. The following results were obtained from cultured cells PHA transformed cells were negative, poly morphs were negative or weakly positive most macrophages were negative, but a few were weakly positive, possibly as a result of ingestion of polymorph debris. In all control slides in which sub-

strate was absent from the incubation medium no granular positivity was seen but the diffuse background stain was still present.

Acid phosphatase In all smears lymphocytes were positive with 1–6 dark brown small discrete granules. In buffy coats 95 / of polymorphs were positive, of which about 20 / were strongly positive. After two days incubation the percentage of positive polymorphs was unchanged but the reaction was much weaker

In smears of incubated cells macrophages were found to be strongly positive, the positive granules being present in the cytoplasm and never in the vacuoles. PHA-transformed cells were also positive with about 10 or more granules. A granule of dye was frequently found in one of the cytoplasmic vacuoles in the 'blast like cell, but a number of vacuoles were negative. An area corresponding to the pale staining perinuclear zone of cytoplasm also gave a positive reaction. In all instances control slides incubated in the absence of substrate were negative.

Non-specific esterase In buffy coats smears the most striking positivity was found in monocytes, most of these cells being positive and some strongly positive. Lymphocytes and neutrophils were negative. Macrophages varied considerably in their reaction, some being very strongly positive, and others negative cells with large vacuoles and little cytoplasm tended to be negative. PHA-transformed cells were all negative, as were incubated lymphocytes and polymorphs. In all instances control slides were negative.

5-Nucleotidase In freshly prepared buffy coat smears neutrophils showed many positive granules eosinophils were negative or weakly positive, and monocytes were mostly positive with a few strong positive and a few negative reactions. Lymphocytes were usually negative, but some cells had 1–3 granules and an occasional cell had several granules. Macrophages were generally positive, and the degree of positivity was roughly proportional to the size of the cell.

Some PHA-transformed cells had a small or moderate number of granules, and there was some tendency for positivity to be localised in cytoplasmic vacuoles in these cells, as in the acid phosphatase reaction.

In the cultures, untransformed lymphocytes were almost all negative, with an occasional weakly positive cell, and polymorphs were either negative or weakly positive. In all instances control slides were negative.

Leucine aminopeptidase A similar reaction was seen in all cells in the form of a granular and diffuse positivity Polymorphs, lymphocytes and monocytes were all positive. PHA-transformed cells were positive and some cytoplasmic vacuoles were positive. Macrophages were positive but their vacuoles were negative. In control slides with the omission of substrate or the presence of an enzyme inhibitor the reaction was negative and only a weak brown diffuse stain was seen.

Succinic and isocitric dehydrogenases. Almost all the lymphocytes in buffy coat smears gave a positive reaction in the form of a number of discrete granules and a weak diffuse stain. Polymorphs were mostly negative but a few showed weak or moderative positivity Monocytes generally showed the same degree of positivity as the lymphocytes but some cells were negative. Platelets were positive. In PHA treated cultures the untransformed lymphocytes and the polymorphs showed the same reactions as their unincubated counterparts. PHA transformed cells were more strongly positive than lymphocytes.

Malic and lactic dehydrogenases. The results were the same as for succinic dehydrogenase with the exception that polymorphs from buffy coat smears were mostly positive.

α-Glycerophosphate dehydrogenase Lymphocytes and monocytes from buffy coats were positive and polymorphs showed either similar or weaker reactions. Most PHA-transformed cells were more strongly positive than lymphocytes macrophages were more variable in their reaction, and while most cells showed the same degree of positivity as lymphocytes, some were less positive and a number were strongly positive.

β-Hydroxybutyric dehydrogenase Polymorphs were negative or weakly or moderately positive in buffy coat smears, and negative after incubation. Monocytes were negative, lymphocytes were positive, and PHA-transformed cells strongly positive. Macrophages varied considerably in their reaction most were moderately or strongly positive, but a few were negative.

Glutamic dehydrogenase Lymphocytes, monocytes and macrophages all showed a moderate degree of positivity Polymorphs were often negative but many showed a moderate degree of positivity even after incubation. PHA-transformed cells were strongly positive.

In all the dehydrogenase reactions described so far control slides showed either no staining or only a weak diffuse background stain. Granular positivity was not seen.

Glucose-6-phosphate and 6-phosphogluconate dehydrogenases. Lymphocytes, monocytes and polymorphs were equally positive in buffy coat smears, but the reaction of polymorphs became weaker after two or three days incubation. PHA transformed cells and macrophages were either equally positive or more strongly positive than small lymphocytes. Poor reactions were obtained when preliminary saline incubation was used, but even when this was omitted, cells in control slides showed only a weak diffuse stain with not more than an occasional granule.

Diaphorases. DPN diaphorase was demonstrated in lymphocytes, macrophages, and PHA transformed cells. TPN diaphorase was also present but the reaction was weaker Control slides incubated in the presence of substrate but absence of coenzyme were negative.

Phosphorylase. Polymorphs from buffy coats showed consider able variation in their reaction with some diffusely positive cells and some negative. A few lymphocytes showed a small number of positive granules in their cytoplasm. Macrophages were mostly negative, with an occasional weak positive reaction, and PHA transformed cells were negative.

Discussion

The nitro-BT method was first used to demonstrate dehydrogenases in leucocytes by DE SOUZA AND KOTHARE (33) ACKERMAN (1) found that neutrophils were positive and lymphocytes rarely positive by this method, and that a reaction occurred even when no substrate was present, although it was greater when succinate was added to the incubation medium. MARCUSE AND COCHRAN (23) obtained positive reactions in the absence of substrate, this again merely demonstrating non-specific activity BALOGH AND COHEN (3) were able to obtain negative results when no substrate was present, and thus able to demonstrate specific dehydrogenases in all types of leucocytes in blood films succinic, lactic, glutamic, β-hydroxybutyric, glucose-6-phosphate, 6-phosphogluconate and isocitric dehydrogenases were shown to be present. Our results agree with this work as far as lymphocytes and monocytes are concerned, but some of the reactions of polymorphs are different. Whilst appreciable positivity has been demonstrated in polymorphs for 6-phosphogluconate and glucose-6-phosphate dehydrogenases suggesting that these cells can use the pentose-shunt pathway only

weak positivity was demonstrated for lactic dehydrogenase and enzymes of the Krebs' cycle, these results being in substantial agreement with HAYHOE *et al.* (19) Biochemical studies (24) showed that cell suspensions in which 90–95 / of the cells were polymorphs were capable of metabolising glucose to lactic acid. FREI *et al.* (14) examined glycolysis in various suspensions of leucocytes in which the proportion of the different cell types varied. By the use of a computer they were able to compare activities of each type of cell, and found that the neutrophil possessed greater glycolytic activity than the lymphocyte.

The enzymatic activity in macrophages in the present study is roughly the same as that of the lymphocyte, as far as glycolytic and Krebs cycle enzymes are concerned, but rather greater than the lymphocyte for enzymes of the pentose-shunt pathway. Using inflammatory cells obtained by the skin window method (31) the presence of succinic, malic and isocitric dehydrogenases has been found in macrophages (40 41) The high degree of positivity of PHA transformed cells for glycolytic and Krebs' cycle enzymes indicates the high potential of energy production that these cells possess.

α-Glycerophosphate dehydrogenase has been found in lymphocytes and macrophages from inflammatory lesions (40) The increased positivity of PHA transformed cells for this enzyme, and for β-hydroxybutyric dehydrogenase indicate an increased degree of lipid metabolism compared with that of the lymphocyte, and correlates with the findings of increased carbohydrate and protein metabolism. The presence of increased activity of glutamic dehydrogenase in PHA-transformed cells is associated with other evidence of protein synthesis (15)

Phosphorylase was first demonstrated in neutrophils by TAKEUCHI AND KINOSHITA (34) who also obtained negative results in lymphocytes and monocytes. WULFF (39) obtained positive results in neutrophils in inflammatory reactions but found that blood neutrophils were negative. QUAGLINO AND HAYHOE (29) using a modified method found that not only were neutrophils positive, but some monocytes and lymphocytes as well. Similar results have been obtained in the present study suggesting that the modified method is more sensitive than the original. This is presumably due to the higher concentration of substrate and the prevention of loss of substrate by glycolysis, by inhibition with fluoride.

Aminopeptidase activity has been demonstrated in polymorphs but not in lymphocytes by ACKERMAN (2) using alanyl β-naphthyl amide as substrate. The finding of aminopeptidase activity in the present work is not necessarily in disagreement with this as a different substrate has been used. The presence of a glycyl-glycine dipeptidase whose activity is greater in lymphocytes than in poly morphs, has been demonstrated (13)

Alkaline phosphatase was first demonstrated cytochemically in granulocytes by RABINOVICH and ANDREUCCI (30) but there was considerable diffusion of positivity in the GOMORI method used by these authors, resulting in false nuclear localisation in neutrophils and positivity in lymphocytes. However using the same method WACHSTEIN (36) found that neutrophils were positive and lymphocytes negative. The more satisfactory azo-dye method showed that positive reactions were restricted to the granulocyte series (18, 21) The present investigations confirm the presence of this enzyme in neutrophils, and the occasional positive result in macrophages can be accounted for by ingested polymorph debris.

Some of the vacuoles in PHA-transformed cells stain positively for acid phosphatase and 5-nucleotidase, and may be lysosomes. These vacuoles correspond in size and position to the dense bodies seen in the electron micrographs of these cells (10 20 35) and also contain neutral fat (15) The high degree of acid phosphatase activity in macrophages may also be associated with lysosomes. These particles have been demonstrated in the alveolar macrophages of rabbits treated with BCG (8) By means of differential centrifugation in a sucrose gradient it was found possible to separate mitochondria from hydrolytic particles and the latter were shown to contain a number of enzymes known to be present in lysosomes.

Strong acid phosphatase positivity in neutrophils and slight positivity in lymphocytes have been demonstrated by the azo-dye method (32) and confirmed in the present investigations. Tissue macrophages show strongly positive reactions for acid phosphatase and non-specific esterase (5, 9)

There was some disagreement in the early results of non-specific esterase activity which is most likely due to the use of different substrates by different authors. When naphthol AS-acetate is used monocytes and polymorphs are positive, and lymphocytes only weakly positive (19 38) Neutrophils are negative when α-naphthyl acetate is used, but monocytes are positive (4) The discrepancies

may be explained if it is assumed that the enzyme acting on the two substrates are different. The present study using α-naphthyl acetate, has demonstrated strong positivity in monocytes and some macrophages, and negative reactions in lymphocytes and polymorphs. In skin-window preparations macrophages are positive when either α-naphthyl acetate or naphthol AS-chloroacetate is used (42)

Wulff (42) concluded that in view of the fact that the macrophage resembled the monocyte in its cytochemical reactions rather than the lymphocyte, macrophages arise from monocytes. This is in direct contradiction to the conclusions based on morphology of cells at various stages of inflammatory reactions (31) and on those obtained from cultural studies by the present authors (16) The use of cytochemistry to determine the probable ancestry of a given cell type is open to criticism. Whilst the macrophage and monocyte possess a non-specific esterase, the absence of this enzyme from the lymphocyte does not necessarily mean that it cannot be synthesized under suitable circumstances. Alkaline phosphatase for example, is demonstrable in neutrophils and metamyelocytes, but not in myeloblasts or myelocytes. This suggests either that the enzyme is not present or that the cytochemical technique is inadequate for its demonstration. In either event it could hardly be argued that a myelocyte does not give rise to a metamyelocyte on this evidence. It seems more likely that the presence of a non-specific esterase in macrophages is a functional adaptation and does not preclude a lymphocytic ancestry However the possibility cannot be excluded that in fluid cultures, macrophages with the strongest non-specific esterase positivity arise from monocytes.

The present studies have therefore shown contrasting metabolic patterns in macrophages and PHA-transformed cells compared with their parent cell, the lymphocyte. Macrophages exhibit a greater degree of certain hydrolytic enzymes than the lymphocyte and a comparable degree of carbohydrate and protein metabolism. PHA-transformed cells are characterised by the presence of a high degree of metabolic activity in terms of carbohydrate and protein metabolism, which is associated with a greater amount of protein and nucleic acid synthesis than the lymphocyte (15)

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Summary

Lymphocytes have been compared with PHA-transformed cells and macrophages derived from leucocyte cultures for various cytochemically demonstrable enzymes. Acid phosphatase and 5-nucleotidase are found in all three cell types, but macrophages show the strongest reactions. PHA-transformed cells stain more intensely than lymphocytes for these enzymes and localisation in some of the cytoplasmic vacuoles is seen. Many macrophages stain strongly for nonspecific esterase while the other cells types are negative. Glycolytic and Krebs cycle enzymes are present in lymphocytes and macrophages to comparable degree, and to a greater extent in PHA-transformed cells. Enzymes of the pentose phosphate pathway are present in lymphocytes and also in the other cell types to an equal or greater degree.

Zusammenfassung

Lymphozyten wurden bezüglich ihres Gehaltes an verschiedenen zytochemisch nachweisbaren Enzymen verglichen mit durch Phytohämagglutinin (PHA) umgewandelten Zellen und mit Makrophagen aus Zellkulturen. Saure Phosphatase und 5-Nukleotidase finden sich in allen drei Zelltypen, wobei Makrophagen die stärksten Reaktionen zeigen. Mit PHA umgewandelte Zellen geben intensivere Färbungen für diese Enzyme als Lymphozyten, wobei eine Lokalisation in einigen der Zytoplasmavakuolen zu erkennen ist. Manche Makrophagen geben eine starke Färbung für eine unspezifische Esterase, während die anderen Zelltypen negativ sind. Enzyme der Glykolyse und des Krebs-Zyklus finden sich in gleicher Weise in Lymphozyten und Makrophagen und in stärkerem Ausmass in durch PHA umgewandelten Zellen. Enzyme des Pentosephosphat-Umsatzes kommen in Lymphozyten vor, ferner in den anderen Zelltypen in gleicher oder grösserer Menge.

Résumé

Des lymphocytes ont été comparés avec des cellules transformées à l'aide de phytohémagglutinine, ainsi qu avec des macrophages dérivés de cultures de leucocytes, quant à leur contenu en différents enzymes démontrables par des méthodes cytochimiques. De la phosphatase acide et de la 5-nucléotidase se trouvent dans les trois types de cellules. Les macrophages montrent les réactions les plus fortes. Les cellules transformées à l'aide de PHA donnent pour ces enzymes des colorations plus intenses que les lymphocytes. Les colorations sont localisées dans quelques unes des vacuoles du plasma. De nombreux macrophages donnent une forte coloration pour une estérase non-spécifique tandis que les autres types de cellules restent négatifs. Des enzymes de la glycolyse et du cycle de Krebs se trouvent à un degré comparable dans les lymphocytes et les macrophages et en plus forte proportion dans les cellules transformées à l'aide de PHA. Les enzymes du cycle du pentose-phosphate sont présents dans les lymphocytes et dans les autres types de cellules en quantités égales ou plus grandes.

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Aconitase Activity in Iron Deficiency

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The enzyme aconitase catalyses the equilibrium between citrate, isocitrate and aconitate. Aconitase needs iron as a co-factor in the conversion of citrate to *cis*-aconitate and *iso*citrate in the tricarboxylic acid cycle (6). In experimental iron deficiency interesting changes were observed in the activity of this enzyme. In the iron deficient rat, the activity was markedly depleted in the kidney but only slightly so in the heart with no change in liver and brain (3). In two patients of iron deficiency anaemia investigated by BEUTLER and YEH (4) aconitase activity in blood per unit of cells did not show any significant variation from the normal pattern. Available literature on aconitase activity of human blood in iron deficiency is scanty. In our preliminary studies designed to assess the aconitase activity of human blood, there were evidences to suggest depressed activity of the enzyme in some cases of iron deficiency (8, 9). The purpose of this communication is to present the results of enzyme assays on whole blood and in different fractions thereof in normals and patients with iron deficiency.

Material and Method

In the *iron deficiency group* there were 39 patients with ages ranging from 2 to 62 years. There were 22 males and 17 females. Haemoglobin level varied from 1.7 to 9.8 g%; in 11 subjects, the value was below 5.0 g%. Thirty patients had hypoferraemia (serum iron levels varied from 14 to 65 µg%) while 9 showed normal values for serum iron. Depletion of haemosiderin from the bone marrow was found in all cases. The cause of iron deficiency was blood loss in 33 (hookworm 22, menorrhagia 5, piles 4, duodenal ulcer 2), nutritional in 5 and post-gastrectomy syndrome in one.

In the *normal group* there were 18 subjects derived from physicians, laboratory personnel, and medical students. Their ages ranged from 25 to 45. None had any history of blood loss. Their haemoglobin level ranged from 13.0 to 17.5 g%. Serum iron levels varied from 85 to 150 µg%.

Aconitase assay. For aconitase assay 3 ml of venous blood was collected using heparin as the anticoagulant. Immediately after collection, the blood was transferred to an ice bath. Whole blood was lysed in ice-cold distilled water and kept in ice bath. Within 10 to 15 min, the sample appeared clear. All the assays were completed within one hour of collection of blood.

Activity of the enzyme in the lysate was determined colorimetrically by following the rate of reduction of the redox dye 2-6-dichlorophenol indophenol in presence of sodium citrate, pig heart extract, triphosphopyridine nucleotide (TPN), and manganese chloride in phosphate buffer medium of pH 7.4 at 30 °C. The rate of reduction was followed in Leitz photoelectric colorimeter at 610 mμ wave length. A suitable substrate blank without citrate and TPN was run simultaneously to eliminate non-specific reduction of the dye. This procedure was adapted from the methods employed by ADLER *et al.* (1), JOHNSON (5), OCHOA (7) and BARRAN and SCHULTZ (2). The unit of activity was taken as the rate of change of optical density per minute under the conditions of the test. The results were expressed in units of activity per ml of whole blood per minute.

All the estimations were done in duplicate. The initial enzyme activity before the institution of iron therapy as recorded for each patient was the average value of two examinations on two different days. There was good agreement between the two values.

In patients, showing low enzyme activity, the effect of addition of iron *in vitro* was studied.

Results

The mean value for aconitase activity in iron deficiency was 1.12 units/ml of blood with a range varying from 0 to 4.3 units/ml of blood per minute. The value was below 0.50 in 7 cases and below 0.75 units/ml blood/minute in 16 cases. The mean normal value was 2.1 with a range from 1.03 to 3.50 units per ml blood. A scatter distribution of enzyme activities in each individual cases both in the normal and in the iron deficiency group is shown in Fig. 1.

Changes in Enzyme Activity Following Iron Therapy

(1) Group with low activity of enzyme. In 16 cases, the value of enzyme activity was below 0.75. In all these cases, haemoglobin level improved steadily with oral iron and attained normal or near normal level during the period of observation. Serial determination of enzyme activity could be made in 11 cases. The enzyme activity usually increased sharply during the reticulocytic peak. The enzyme activity decreased as the reticulocyte count declined thereafter the activity gradually increased in 10 cases. The activity became normal in 8 and was partially improved in 2 cases. Serial changes in two cases in which the enzyme activity became normal are shown in Fig. 2 and 3. In 1 case, no improvement of enzyme activity was noticed.

Fig 1 A scatter distribution of enzyme activities in each individual case in the normal and in the iron deficiency group.

(2) Group with normal activity of enzyme In 23 cases, the enzyme activity was within normal range. Except for fluctuation related to the reticulocyte level, no significant change was noticed in the serial values of enzyme activities.

Aconitase Activity and Blood Values

There was no significant correlation between the enzyme activity and the quantitative level of white cells, platelets and red cells of the patient as observed initially before treatment or finally after improvement and normalisation of haemoglobin level. Haematological data in two groups of patients are shown in Table I

During the reticulocyte phase, the activity was found to increase in general. Rate of haemopoietic response did not also appear to have any relationship with level of enzyme activity

Effect of addition of iron in vitro on the activation of aconitase activity. One gamma of elemental iron (as ferrous sulphate) was added per ml of ice-cold blood lysate diluted 1 10 and incubated at 4 °C for 30 min. Addition of iron failed to restore the low enzyme activity

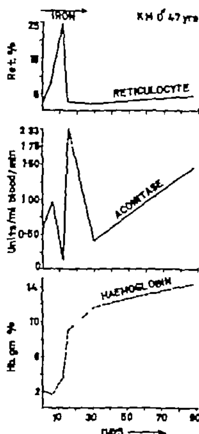


Fig. 2. Serial data during iron therapy on hemoglobin level, aconitase activity and reticulocyte count in 47-year-old male patient. The iron deficiency was due to blood loss consequent on hookworm infestation. The initial enzyme activity was low. During reticulocytic peak, the enzyme activity increased sharply. The activity decreased as the reticulocyte count declined. Thereafter along with normalisation of hemoglobin level the activity gradually attained normal level.

Table I

Haematological data in two groups of patients.

	Group with low enzyme activity (14)		Group with normal enzyme activity (27)	
	Mean	Range	Mean	Range
Hb g%	5.5	2.0—8.5	6.1	1.7—8.8
PVC %	21.8	10.0—30.0	23.5	8.0—32.0
WBC/mm³	8 100	3 600—10 400	7 900	2 200—18 400
Serum iron µg%	41.4	9.0—63.0	56.4	10.0—100.0

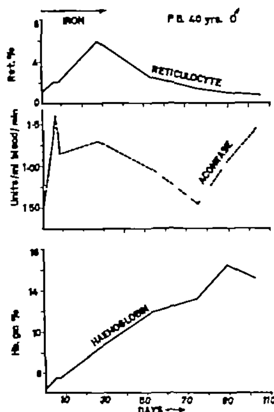


Fig 3 Serial data during iron therapy on reticulocyte count, aconitase activity and hemoglobin level in a 40-year-old patient with heavy hookworm infestation. The following features are illustrated ( ) increase of enzyme activity during reticulocytic peak (b) gradual restoration of enzyme activity to normal level.

Aconitase Activity in Different Fractions of Blood

For this study 10 ml of venous blood was collected in a plastic test tube containing heparin as the anti-coagulant. The tube was kept in a crushed ice bath for half an-hour Red cells, buffy coat (white cells plus platelets) and plasma were separated by differential centrifugation at 4 C using a standard refrigerated centrifuge machine. Red cells were resuspended in saline giving PCV value of 20–30 %. This suspension contained less than 1000 WBC/mm³ and was used for assay of aconitase. The test tube containing WBC and platelets was centrifuged at 2500 rpm for half-an-hour The button of WBC and platelets was suspended in saline. The activity of aconitase as obtained in different layers is shown in Table II.

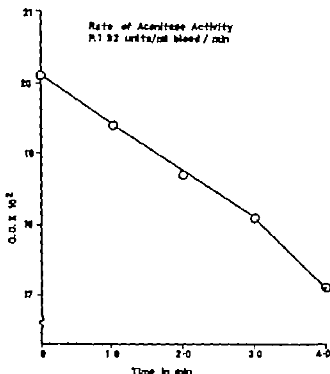


Fig 4 Linearity of aconitase activity during first 3 min when measured with the dye concentration in 2,6-dichlorophenol-indophenol assay

Table II
Aconitase activity (per minute) in different fractions of blood.

Subjects	PCV% of suspension	Units/ml WBC suspension	Units/10^8 WBC (in buffy coat)	Units/ml plasma
GP (ID*)	21	4.4	0.05	0
PS (ID)	24	2.4	0.069	0
JBC (N)	35	3.9	0.184	0
SS (N)	23	1.5	0.277	0

Iron deficiency Normal

Study on Linearity of Enzyme Activity

The linearity of enzyme activity is shown in Fig 4 where the optical density after correction of endogenous blank is plotted against time in minutes after making necessary blank corrections. The activity was found to be linear for the first 3 min and showed a decline thereafter in the majority of cases.

Aconitase Activity and Clinical Symptoms

The clinical features did not in general appear to have any correlation with the initial enzyme activity. The only patient in whom the enzyme activity remained low was a 45-year-old male with heavy hookworm infestation. His haemoglobin level became normal but his clinical symptoms were not completely relieved: he still complained of considerable asthenia and myalgia. Considerable symptoms persisted also in one of the two patients in whom the enzyme activity was partially improved.

Discussion

Due to obvious difficulties in obtaining pure cell suspensions, it was thought that aconitase activity of whole blood could be conveniently utilised in assessing the enzymatic status in iron deficiency and in following its behaviour during treatment. In a series of 39 cases of iron deficiency anaemia of varying grades of severity the activity as calculated in units per ml of whole blood was low in 16. The activity was low even when expressed per ml of cells. In the remaining 23 the values were within normal range. Though direct correlation of the enzyme activity with any of the haematological parameters was not obtained, it was noticed that low enzyme activity was almost never associated with normal serum iron values. The converse was not, however, true: hypoferraemia was not always associated with low enzyme activity. Experiments designed to analyse the activity in different fractions indicated significant activity both in the red cells and in the buffy coat. In iron deficiency the activity in the buffy coat was grossly depleted while red cells showed normal values. The plasma was devoid of activity both in normals and in iron deficiency subjects.

In the group with low initial enzyme activity, serial studies in 11 patients during iron therapy indicated that the aconitase activity was elevated during the reticulocytosis phase. The fall in the reticulocyte level was accompanied by decline in the enzyme activity to levels below normal. Thereafter the activity gradually rose and became normal in 8, partially improved in 2 cases and remained unaltered in one. In an individual patient, the enzyme activity could not be correlated with levels of red cells, haemoglobin, white cell and platelet. The observed activity may therefore be taken as an expression of overall change in the red cells, white cells and platelets.

The exact mechanism of the depression in the activity of aconitase as observed in 16 out of 39 cases is not known. The improvement in enzymatic activity after iron therapy indicates the possibility of iron deficiency being in some way contributory. The failure of reactivation of the enzyme after *in vitro* addition of iron indicates that under conditions of iron deficiency the production of the enzyme proper was depressed. The depressed activity was not due to mere absence of iron and consequent interference in the activation by the co-factor. These findings were similar to those recorded by BEUTLER in iron-deficient rat kidney (3). In the absence of precise knowledge regarding the factors responsible for the production of aconitase deficiency it is not possible to state as to why the deficiency was not demonstrable in all the cases.

Lastly the role of aconitase deficiency if any in the production of clinical signs and symptoms need be considered. No significant correlation could be found between the enzyme activity and the following parameters: degree of anaemia and hypoferraemia, presenting clinical features, rate of haemopoietic response to iron therapy and the basic cause of iron deficiency. From the persistence of clinical symptoms as recorded in one patient with persistently low enzyme activity and also in one of two patients in whom the enzyme improved only partially it would be tempting to correlate the relevant symptoms to aconitase deficiency. But in an overall appraisal of the general findings, and in view of the limited data so far available, no definite conclusion would appear to be warranted.

Summary

The activity of the enzyme aconitase in whole blood was determined by following the rate of reduction of the redox dye 2-6-dichlorophenol indophenol in the presence of sodium citrate, pig heart extract triphosphopyridine nucleotide, manganese chloride in phosphate buffer of pH 7.4. The mean normal value was 2.1 with range from 1.03 to 3.5 units per ml of blood. In iron deficiency the mean value was 1.12 with range from 0 to 4.3; the value was below 0.5 in 7 and below 0.75 in 16 cases. A sharp rise in the enzyme activity was noted during the reticulocytic peak following iron therapy. With the subsidence of reticulocytosis the activity declined. Thereafter along with improvement of haemoglobin level, the activity gradually rose and attained normal levels in 8 of the 11 subjects studied serially; the activity was partially restored in 2 and unaltered in one.

Zusammenfassung

Im Vollblut wurde die Aktivität von Akonitase untersucht durch Bestimmung der Reduktionsrate des Redoxfarbstoffes 2,6-dichlorphenol-indophenol in Gegenwart von Natriumzitrat, Triphosphorpyridinnukleotid aus Schweineherzextrakt und Mangan-

chlorid in Phosphatpuffer von pH 7,4. Die Normalwerte betrugen im Mittel 2,1 Einheiten pro ml Blut mit einer Streubreite von 1,03–3,5. Bei Eisenmangel betrugen der Mittelwert 1,12 E. und die Streuung 0–4,3; der Wert lag unter 0,5 bei 7 und unter 0,75 bei 16 Fällen. Ein steiler Anstieg fand sich während der Retikulozytenkrise nach Eisentherapie. Die Aktivität nahm ab mit dem Rückgang der Retikulozytose. Anschließend nahm die Aktivität entsprechend der Besserung des Hämoglobingehaltes allmählich zu und erreichte normale Werte bei 8 der 11 untersuchten Patienten; sie wurde teilweise wiederhergestellt bei 2 und blieb unverändert bei einem Patienten.

Résumé

L'activité de l'aconitase a été déterminée dans du sang complet en mesurant la vitesse de réduction à l'aide du colorant 2-6-dichlorphénol indophénol en présence de citrate de sodium, de triphosphopyridine-nucléotide venant d'extraits de cœur de porc, de chloride de manganèse dans un tampon de phosphate au pH de 7,4. La valeur moyenne normale était de 2,1 unités. Dans les états déficients en fer, la valeur moyenne était de 1,12, la dispersion de 0 à 4,3; la valeur était en dessous de 0,5 dans 7 et en dessous de 0,75 dans 16 cas. Une forte augmentation de l'activité enzymatique fut notée durant la crise réticulocytaire suivant un traitement au fer. L'activité diminua avec la baisse de la réticulocytose. Par la suite l'activité augmenta graduellement avec l'amélioration du taux d'hémoglobine et atteint des valeurs normales chez 8 de 11 personnes examinées en série; l'activité était partiellement rétablie chez deux malades et resta inchangée chez un.

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Fundación Hematológica de Mar del Plata

Agglutinin Synthesis Regulation Independent of Antigen

E. Rewald and C. Pellitero

It has been shown that the antibody response diminishes when massive doses of homologous serum are injected during the period of sensitization (Substitutive-inhibitory treatment) (1) The pur pose of the present brief communication is to describe an experiment planned to determine whether on the other hand massive plasma pheresis enhances the agglutinin titre of rat serum against sheep erythrocytes.

Methods

Random mated female rats fed with 'Forrumez and water *ad libitum* were housed t 21–23 °C in individual cages. 50% of each litter was placed in the experimental group, and both groups were sensitized at the age of one month with sheep erythrocytes (washed and resuspended) by intraperitoneal inoculation. These injections were given on alternative days in three initial doses of 0.3 ml and two of 0.1 ml as has been described previously (1). Coincident with the first sensitizing injection, blood was extracted (following ether anaesthesia) by cardiac puncture, an amount equivalent to 2.5% body weight *per dose* on 9 subsequent occasions. The rats were left for 48 h prior to determination of the antibody titre. After each cardiac puncture, similar amount of washed red cells resuspended in saline was injected by the intraperitoneal route. As negative controls, *identical blood extractions were carried out, but the whole blood was returned* immediately by the intraperitoneal route. The mortality rate during this experiment was 76.47% in the experimental rats and 54.4% in the control animals.

Most of the surviving animals showed relatively good condition when the antibody titre was determined.

Most animals died during or immediately after a heart puncture. High mortality does not depend exclusively on the frequent heart punctures plus sudden removal of large amounts of blood it seems to be mainly technical problem since the rate diminished progressively In the last litters the mortality of experimental and control animals was equal.

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Results

As shown in the table the agglutinin titre of the experimental rats is significantly increased* As during massive plasmapheresis a certain amount is lost, it has to be supposed that the true increase of the titre would be even higher It is of special interest to note that the titre of the controls, upon which daily cardiac blood extractions and intraperitoneal returns were performed was similar to the one obtained for the saline inoculated control animals during an experiment described previously (1)

Agglutinin titre (Log_2)	Agglutination 14 exp. rats	21 control rats
5	100%	100%
6	100%	90%
7	100%	43%
8	57%	4.8%
9	35.7%	4.8%
10	14.3%	—
A	8.07±0.20	6.43±0.13
	$p<0.001$	

Discussion

We assume that agglutination is due mainly to γM. Our aim is to limit the experimental model to the intravascular compartment in order to avoid that variations of distribution and/or fractional catabolism interfere the results (2,3)

Our results present further (4) evidence to show that independent of the antigenic stimulus, the gamma globulin synthesis may depend also on a distinct regulatory mechanism. Employing the same antigenic stimulus, it was possible to modify the titer in a range of nearly 500 / by adding or removing serum during the sensitization period.

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* Serum dilutions were performed in saline.

Summary

Massive plasmapheresis during the sensitization period enhances significantly the agglutinin titre of rat serum against sheep erythrocytes. These results suggest that independent of the complex antigenic stimulus, the gamma globulin synthesis may depend also on distinct regulatory mechanism.

Zusammenfassung

Durch Entzug von grösseren Plasmamengen während der Sensibilisierung von Ratten gegen Schaferythrocyten wird der Agglutinintiter signifikant erhöht. Dieses Ergebnis wird als weiterer Hinweis gedeutet, daß, unabhängig vom Antigenstimulus, die Gamma-Globulinsynthese durch einen weiteren Mechanismus reguliert wird.

Résumé

La plasmaphérèse faites en grandes quantités pendant la période de sensibilisation accroît de manière sensible le taux d'agglutinines sériques du rat envers l'érythrocyte de mouton. Ces résultats suggèrent que, indépendemment du stimulant antigénique, la synthèse des gamma-globulines pourrait dépendre encore d'un autre mécanisme régulateur.

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Erythrokinetic Studies in Thalassemia with Simultaneous Radioactive Tracers (Fe^{59} and Cr^{51})*

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Thalassemia is actually regarded as an inherited haemolytic anaemia resulting from an intracorpuscular defect. Studies on the erythropoietic activity on red cell destruction and on iron metabolism have been undertaken to explain the nature of thalassemia (1–15) A reduction of life-span of erythrocytes was demonstrated in thalassemia major (2, 3 5–7 9 13 16–20) and it is also suggested that a high proportion of red cell precursors in the bone marrow may not deliver in viable form to the circulating blood (5 7 13)

The anaemia of thalassemia appears to be due to impaired erythropoiesis and in part to increased red cells destruction. A defect in haemoglobin synthesis and a premature destruction of the red cell precursors within the bone marrow has been shown in thalassemia with the use of ^{14}C-glycine *in vivo* (10 12) A block in the heme synthesis localized at the stage from glycine to Ala has been suggested (10 21) but recently it has been demonstrated that the globin synthesis is selectively deficient in thalassemia major (22, 23) and minor (24–26) while the depression of heme synthesis may be determined by a secondary phenomenon (27 28) only in thalassemia major (24) But the exact nature of the defect of erythropoiesis in thalassemia remains unexplained so as the reason or erythrocyte life-span reduction.

Simultaneous studies of erythropoiesis (iron utilization, hemoglobin synthesis and erythropoiesis *in vivo*) and red cell destruction (red cell survival and identification of sites of red cell destruction)

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may be possible with the use of simultaneous radioactive tracers (Fe^{59} and Cr^{51}) (18, 29) The present study reports our observations using this double radioactive method in children with thalassemia major these researches were undertaken to define further relationship between erythropoiesis and haemolysis in thalassemia in relation to clinical status, to age and to haematological findings.

Methods and Materials

W have studied 24 Sardinian children affected by thalassemia major classificated according to BALANDSON et al. (7) Three children had already been splenectomized for long time. Two children were splenectomized after the study they were examined again eight and ten months. The children had not been transfused (No. 1 3 8, 9 14–16, 22 25, 27) or had received no transfusion for 4 months prior of the experiment.

Haematological methods. Counts for red blood cells were performed in the automatic high speed Counter "Celloscope" Haematocrit was made with capillary tubes (Cellocrit, Ljungberg) Haemoglobin determinations were made according to DRABKIN's method. Osmotic fragility of red cells was assayed with screening procedure according to MALAMOS et al. (30) Reticulocyte counts were made on dry films after vital staining with Brillantkresylblau t 37 °C. Determinations of percentage of foetal hemoglobin were performed with alkali denaturation method (SINGER et al., 31) and percentage of haemoglobin A_2 was determined by starch block electrophoresis according to KUNKEL and WALLENIUS (32) Serum iron concentration and total iron binding capacity were estimated according to the methods of RAMSAY (33, 34) Bilirubin concentration was done by spectrophotometric method of ENGELHARDT (35) The sickling phenomenon was investigated with the use of reducing substance (sodium dithionite) The other haematological investigations were carried out with the conventional methods.

Radioisotope methods. Simultaneous radioactive tracer studies were carried out.

A sample of the patient' red cells of 10 ml was collected in a sterile bottle with 2.0 ml of acid-citrate dextrose solution and with Chromium^{51} (as sodium chromate with high specific activity 96 $\mu c/mg$) (The Radiochemical Centre Amersham) in doses of 3 $\mu c/kg$ The mixture was incubated at 37 °C for 30 min and the reaction then terminated by addition of 100 mg of ascorbic acid.

Iron^{59} (as ferric citrate, specific activity of 7 $\mu c/\mu g$) (The Radiochemical Centre Amersham) was added to 12 ml of plasma of normal donor in amount of 1.0–1.2 μc and incubated for 30 min at 37 °C to ensure complete binding of iron. An exact amount of plasma-Fe^{59} was retained for the determination of standard activity

The erythrocytes labeled with Cr^{51} were autotransfused 35 min after the injection of plasma-Fe^{59} The amounts of erythrocytes tagged with Cr^{51} injected were exactly determined. A sample of whole blood diluted 1 100 and sample of plasma diluted 1:25 were collected for the counts.

Sample of heparinized blood was withdrawn after 10, 35 and 60 minutes and every two days for almost 20 days. The counts were made on the plasma and on the whole blood.

The surface counts *in situ* were performed after 5 25 and 45 minutes and each time the blood was sampled, in marked positions on the sacral bone marrow on the liver on the spleen and on the armpit with directional scintillation counter with crystal of NaJTl 1½ × 1½ and well type collimator operated in conjunction with superscaler with pulse high analyzer (DCS 45AU SELO Milano)

The samples of blood and of plasma were counted in well type scintillator counter t the end of experience for total of 10000 counts. The samples were counted

in duplicate before for the Cr^{51} and after for the Fe^{59} The observed counting rates for Cr^{51} were then corrected for the small contribution of the Fe^{59} counts of the samples. The rates of efficiency of the counts for the Fe^{59} under the operating conditions for measurement for Cr^{51} to that under the conditions for Fe^{59} have been determinated by measurement of the Fe^{59} standard with the pulse high analyzer adjusted to the setting for Cr^{51} and respectively for the Fe^{59}

Also the surface counts for Cr^{51} were corrected for the contributions due to Fe^{59} The data were the further corrected for variations due to radioactivity decay and subtracting from the corrected counting rates the contribution from the circulating blood radioactivity

The results of studies with Fe^{59} were calculated according to the methods of VEALL and VETTER (36).

The Cr^{51} content of the blood samples were plotted on the semi-logarithm scale and the half survival time was estimated for the extrapolation at zero time. The data were not corrected for the elution (29)

Blood volume was calculated by Cr^{51} method and plasma Fe^{59} method.

The circulating Fe^{59} in red cells was calculated as per cent of the dose injected.

Erythrokinetic data were calculated according to the formulas used by MCCURDY (14)

The surface counts were plotted on the linear scale as counts per minute.

The synthesis of haemoglobin was calculated with the ratio 100 g of Hb = 0.338 g of Iron (37).

The spleen localization index was calculated as ratio of counts per minute of spleen/counts per minute of liver at the time of half-survival time of Cr^{51} tagged red cells.

W have assayed in some subjects the radioactivity of whole blood and of stroma-free haemolysate prepared with precipitation with toluene and centrifugation t 32000 x G/20 min. The results were expressed as counts per minute per g of haemoglobin.

Results

The haematological data are referred in the Table I. Results of the studies with Fe^{59} and Cr^{51} are summarized in the Tables II, III and IV The results are referred to normal values based on the data published by BOTHWELL *et al.* (38) and FIGUEROA and WEINSTEIN (39) and for Cr^{51} half survival time by MOLLISON (40)

Plasma Fe^{59} half clearance time has been greatly reduced (values from 50 to 15 min) in splenectomized subjects the values were from 54 to 15 min (Fig 1) The plasma iron clearance curve is not linear in 16 subjects (Fig 2) In these cases we have observed an early and characteristic dip of the plasma radioiron curve at 35 min followed by a prompt rise. In the cases 26, 27 29 and 4bis the plasma radioactivity was counted for several days and a second dip of the curve has been observed at 24 h (Fig 3 and 4)

The net *red cell incorporation of radioiron* shows three different patterns (Fig 5) Type I red cell Fe^{59} uptake curves depressed but of normal shape with a definite plateau (No. 1 2, 3 4 5 6 9 and

Table I
Haematologic data.

No.	Sex	Weight, kg	Age, years	Packed cell volume, %	Plasma volume, ml/kg	RBC mass, ml/kg	Haemoglobin, g %	RBC 10^6/mm³	MCV, μ³	MCH, γγ	MCHC, %	Fetal haemoglobin, %	A_2 haemoglobin of Hb A, %	Free bilirubin, mg %
1	M	16.5	7	23	74.3	23.0	8.8	3.63	68	24	36	68	—	—
2	M	17.0	6	17	83.2	14.7	3.8	2.20	77	17	22	29	—	—
3	F	10.8	5	19	58.1	11.4	5.0	2.50	71	20	26	48	—	—
4	F	15.0	4	19	54.0	11.4	6.2	2.58	73	25	32	80	15.7	3.80
4bis*		16.5	5	15	60.4	19.0	4.2	1.98	73	21	28	73	—	1.63
5	M	20.4	10	27	68.2	21.6	8.6	3.68	73	24	31	63	2.59	0.49
6	M	7.4	1	11	73.1	8.8	3.4	1.64	67	21	31	86	—	—
7	M	9.5	2	12	87.8	10.5	3.0	1.80	68	16	23	7	4.38	—
7bis		10.5	2	10	86.9	8.1	3.4	2.10	41	16	34	9.5	—	0.96
8	F	27.0	10	29	62.9	19.8	9.0	3.84	76	23	31	66	4.92	1.23
9	F	12.5	4	10	66.1	7.4	2.8	1.50	66	18	28	68	3.77	0.26
10	F	7.7	1	24	55.0	16.1	3.6	3.30	72	16	23	54	3.17	0.74
11	M	18.0	7	18	59.8	11.9	3.6	2.08	60	21	31	0.5	1.17	0.76
11bis		21.4	9	18	73.4	16.1	4.9	2.47	72	20	27	43	—	—
12	M	12.0	2	17	62.8	15.4	3.5	1.98	85	19	21	7.5	4.76	0.32
13	F	12.8	3	12	69.7	9.4	4.3	1.60	75	30	35	54	7.23	0.24
14	M	9.3	3	16	56.8	10.7	4.4	2.22	72	20	27	69	6.63	0.38
15	F	4.1	3 mths	18	53.9	7.4	5.8	2.52	72	23	32	57	5.57	—
18	M	15.0	7 mths	15	59.1	8.9	4.8	2.03	75	29	38	38	3.80	0.79
20*	F	17.4	6	22	71.1	18.7	8.0	2.97	75	27	36	27	3.88	0.76
21	M	15.1	3	11	86.8	9.6	3.2	1.50	73	21	29	17	–	0.09
22	M	10.5	2	20	63.6	16.4	3.4	2.80	71	25	27	73	7.20	0.47
24	M	10.1	2	12	73.8	9.4	4.0	2.10	59	19	33	37	2.94	0.70
25	F	16.5	7	9.5	94.1	10.4	2.6	1.40	67	18	27	64	—	0.99
26	M	10.5	2	12	73.3	8.2	3.2	1.80	66	17	26	50	7.92	1.04
27	M	11.0	3	18	83.6	18.4	3.7	2.50	72	14	20	80	—	1.00
29	M	20.0	8	10	77.0	10.0	2.1	1.08	50	20	20	62	—	0.70

* Splenectomized.

Table II
Production indices.

Reticulocytes	Production indices PITR	RBC iron utilization %	Cr^{51}	Daily Hb synthesis g/day	g/l of blood	Destruction Index Cr^{51}	Compensation Index C[...]
	0.68 0.22	80/ 100	0.24		1.0/1.6		[illegible]
4 61	3.75	50	1.29	9.08	5.61	1.59	[illegible]
0.55	3.95	55	1.83	11.10	6.56	3 48	[illegible]
0.88	5.17	33	0.79	5.31	4.53	1.51	[illegible]
2.34	5.16	7	2.04	1.06	1.08	3.00	[illegible]
0.85	7.30	100	3.50	12.70	9.70	6.14	[illegible]
1.54	2.21	33	1.79	4.12	1.82	2.30	[illegible]
0.36	4 44	19	1.37	1.31	2.50	4.42	[illegible]
1.60	—	—	1.75	—	—	4.60	[illegible]
0.33	5.22	100	2.00	5.44	5.55	6.14	[illegible]
2.52	3.99	100	2.34	3.61	1.61	3.28	[illegible]
0.38	3.74	17	1.50	1.73	1.88	3.22	[illegible]
3.60	4.56	20	—	1.02	1.39	—	
0.56	2.06	36	3.25	2.51	1.94	8.31	[illegible]
11.00	4.14	31	—	11 10	5.80	—	
2.18	1.87	97	—	0.65	0.65	—	
1.51	1.87	50	—	1.81	1.90	—	
0.36	2.41	47	—	1.80	2.86	—	
2.00	—	—	1.00	+	—	3.83	[illegible]
1.00	4 77	100	0.34	3.31	9.62	4.40	[illegible]
1.20	2.96	100	1.37	41.30	27.00	2.30	[illegible]
0.25	10.80	100	3.41	33.30	26.30	9.83	[illegible]
1.52	9.76	100	1.52	11 10	12.80	4.41	[illegible]
4.53	10.20	100	2.49	18.40	21.80	7.21	[illegible]
1.08	0.50	100	4.13	24.50	14.80	11.70	[illegible]
2.40	1.21	100	2.94	28.10	36.00	9.97	[illegible]
2.61	4.31	100	4.32	17.00	14.20	6.14	[illegible]
2.33	4.05	100	2.12	32.20	18.40	4.60	[illegible]
				mean	9.46		
				SD	5.71		

10) type II curves depressed both with the maximum of uptake in the first day and then a slow fall (No. 11 11bis and 13) type III red cell Fe^{59} uptake with the maximum of uptake (100 /) in the first days and with a progressive fall (curve like to the curves of haemolytic conditions) (No. 7bis, 8, 12, 16 20 21 22, 24 25, 26 26 27 29 and 4bis) The curves of second and third type are strictly related to *non linear comportament of the clearance curve of* radioiron. In splenectomized children the utilization of Fe^{59} by red cells range from 0.33 to 1 00 / with curves of three types. It is remarkable that the case 4 had a curve of radioiron utilization of

Table III
Red cell survival.

No.	Reticulocytes %	T½ Cr^{51} days	Mean cell life (calculated) days	Splenomegaly	Spleen localization index
1	7.4	24	72	+++	2.27
2	1.5	15.5	33	+++	3.70
3	2.0	24.5	76	++	2.36
4	3.8	12	23	+++	2.16
4bis	3.0	9	16.8	splenectomized	
5	1.5	20	50	splenectomized	
6	2.5	15	26	+	1.16
7	5.0	13	25	+	1.66
7bis	2.0	9	16.8	++	3.02
8	4.0	16	35	+++	2.92
9	2.5	11	20.5	+++	1.79
11	1.5	8	13.7	++++	3.20
12	6.5	—	—	splenectomized	
13	5.0	14,5	30	+	0.83
16	3.0	13	25.8	++	2.93
20	2.5	20	50	splenectomized	
21	1.0	7	11.7	++	2.79
22	4.0	15	25.8	++	1.65
24	13.0	9	16.8	+++	2.97
25	5.0	6	9.8	+++	3.70
26	8.0	7	11.7	+++	4.97
27	7.0	9	16.8	++	4.85
29	7.0	13	25.8	+++	4.50
mean value		13.0			
SD		±5.2			

type II before the splenectomy and a curve of type III eight months after the splenectomy

Plasma iron⁵⁹ turnover as mg/kg/h results very high the mean value was 180.3±63.8 with a range from 38.9 to 450.6 (Table IV)

Red cell iron⁵⁹ turnover expressed as mg/kg/day was increased and the values range from 0.45 to 10.8 with a mean value of 3.36 ±1.50 (Table IV)

Red cell iron⁵⁹ renewal express as per cent/day show high values The mean value was of 28.56±21 49 / /day (Table IV)

Circulating red cell iron⁵⁹ was low the mean value was of 13.7 ±3 0 mg/kg with a range from 9.2 to 26 7 mg/kg (Table IV)

Circulating plasma iron⁵⁹ was high the mean value was of 114.2±60.8 μg/kg with a range from 49.2 to 402 0 μg/kg (Table IV)

Haemoglobin synthesis was calculated as total daily haemoglobin synthesis and as production of haemoglobin relating to blood

Table IV
Iron metabolism.

No.	Serum iron μg/100 ml	Iron binding capacity (μg/100ml)	T½ Fe^{59} min	Red cell iron concentration, mg %	Circulating red cell iron, mg/kg	Circulating plasma iron μg/kg	Plasma Fe^{59} disappearance rate, constant K_1	Plasma Fe^{59} turnover μg/h/kg	Red cell iron[59] utilization, %	Red cell iron[59] turnover mg/day/kg	Red cell iron renewed %/day	Plasma iron turnover rate: mg/100ml
1	120	—	24	118	26.7	89.1	1.73	154.2	50	1.84	6.96	3.75
2	100	—	21	123	18.1	85.1	1.98	168.5	55	2.22	12.20	3.05
3	121	—	19	99	14.2	89.9	2.23	200.6	55	1.09	11.80	3.17
4	215	110	34	111	12.6	116.0	1.23	142.9	7	0.24	1.90	5.16
4bis	295	30	33	81	11.8	199.8	1.28	251.6	100	6.50	32.70	7.30
5	165	465	34	107	23.3	114.0	0.77	87.7	35	0.09	2.18	2.21
6	295	66	39	121	9.8	213.0	2.31	152.1	19	0.70	7.02	4.44
7bis	155	310	36	115	9.4	100.0	2.77	74.5	100	1.78	19.00	5.22
8	150	60	18	108	21.4	94.1	0.99	188.0	100	4.51	2.12	5.99
9	175	290	42	91	13.8	116.0	1.92	115.0	17	0.47	3.40	3.74
10	88	340	22	78	12.6	49.2	0.83	91.5	20	0.45	3.57	4.56
11	112	68	50	105	12.5	67.0	0.80	53.9	36	0.48	3.81	2.06
11bis	128	—	22	91	14.6	92.9	2.56	236.1	31	1.75	12.40	4.14
12	137	298	52	86	13.2	86.1	1.10	69.3	97	1.73	12.90	1.87
13	81	114	38	120	11.4	56.4	0.97	38.9	50	0.46	4.01	1.87
14	105	381	43	88	9.4	39.7	1.13	58.2	47	0.63	0.93	2.41
16	112	—	37	133	11.9	84.1	0.59	95.3	100	2.23	19.10	4.77
20	178	72	15	127	21.3	126.0	1.92	343.1	100	8.27	38.10	2.06
21	270	—	22	98	9.4	402.0	2.16	450.6	100	10.80	114.00	10.80
22	108	—	20	99	16.2	109.0	0.74	235.2	100	5.63	38.10	8.76
24	126	84	13	133	12.5	93.0	2.10	257.7	100	6.17	40.10	10.20
25	263	20	20	92	9.2	100.0	2.31	212.0	100	5.08	54.10	0.50
26	250	30	18	85	7.0	68.0	1.16	388.5	100	9.23	132.00	1.21
27	137	133	28	68	12.5	122.8	1.61	197.8	100	4.75	41.90	4.31
28	182	35	36	69	8.9	124.1	1.92	239.8	100	5.71	64.10	4.05
mean			31		13.7	114.2	1.56	180.3	67	3.56	28.56	4.45
SD ±			7		3.0	60.8	0.86	63.8	10	1.50	21.49	1.76
Normal values			70/ 110					18.3/ 0.8	80/ 100	0.38/ 0.02	0.90/ 1.80	0.68/ 0.22

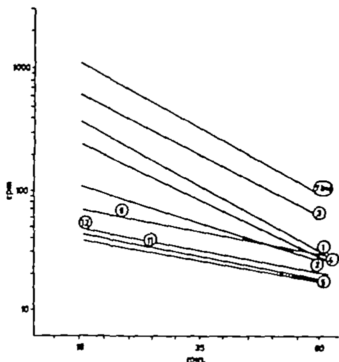


Fig. 1 Plasma clearance curves of tracer dose of iron^{59} linear comportment.

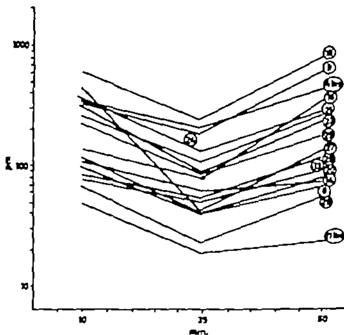


Fig. 2. Plasma clearance curves of tracer dose of iron^{59} non linear comportment.

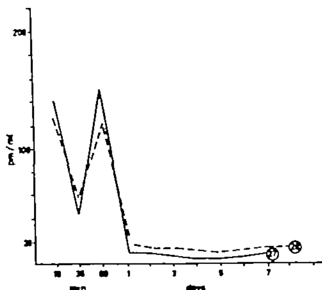


Fig. 3. Plasma Fe^{59} disappearance in two patients with thalassemia major (No. 26 and 27).

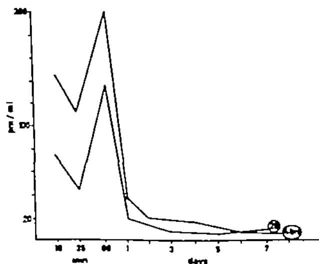


Fig. 4. Plasma Fe^{59} disappearance in two patients with thalassemia major (No. 29 and 4bis).

volume (Table II) The *daily haemoglobin synthesis* appeared to be increased in all subjects values range from 0.65 to 41.30 g The *haemoglobin production relating to blood volume* was in mean 9 46±5 71 g/l with a range from 0.65 to 36.0 g/l of blood. The *survival of* Cr^{51}

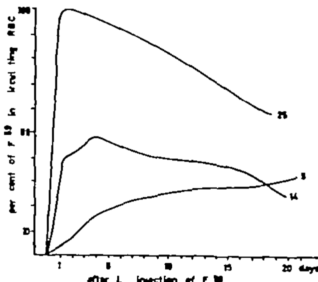


Fig. 5. Curves of appearance of Fe^{59} in the peripheral red cells in thalassemia major

labeled erythrocytes was shortened with mean value of 13.0 ± 5.2 days (Table III).

Red cell production as predicted from the replacement of haemolysed Cr^{51} tagged red cells was increased in all cases (Table II).

The *surface counting studies for Cr^{51}* reveal a marked accumulation of Cr^{51} in the spleen and rather less in the liver as the labelled cells are progressively destroyed in many cases (No. 2, 3, 4, 7, 7bis, 8, 9, 11, 21, 24, 25, 26, 27, 29). The spleen/liver ratio was elevated at the time of the half-survival time of tagged red cells (Fig. 6).

In five cases (No. 1, 6, 15, 16, and 22) the countings over spleen and liver rose simultaneously and the spleen/liver ratio remained constant.

In three subjects with thalassemia major splenectomized (No. 4bis, 5 and 20) the studies *in vivo* with the Cr^{51} show a rise of counting in the liver with the accumulation of the excess of Cr^{51} in the organ (Fig. 7).

The *surface counting studies for Fe^{59}* show diverse pictures. It is necessary to examine individual cases in detail. In three cases (No. 4, 6 and 7) the surface counting over the sacrum bone marrow rose very rapidilly and remained high. Three splenectomized children showed the same aspects of the surface counts (No. 4bis, 12 and 20) but the plasma radioiron clearance was strongly reduced with a non linear curve and the uptake of Fe^{59} by red cells

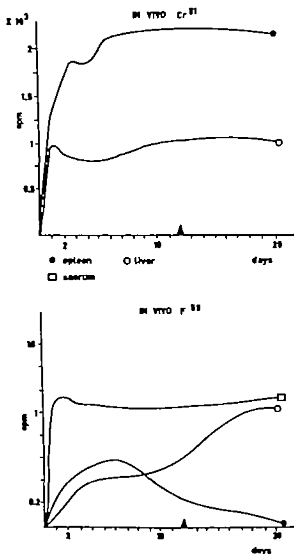


Fig 6. In vivo counting pattern over different body sites after administration of Cr^{51} and Fe^{59} (No. 4, Th. major).

was of 100 / of the dose injected (Fig 7) In the cases No. 9 and 10 we have observed a picture of linear curve of plasma iron clearance, of utilization of Fe^{59} by erythrocytes of moderate elevation of counts on the liver with a constant level and with counting rate over the sacral bone marrow that remains very high after the maximum in the first day A non linear curve of clearance of plasma radioiron,

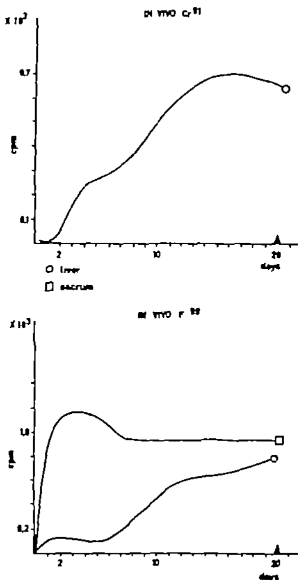


Fig. 7 In vivo counting pattern over different body sites after administration of Cr^{51} and Fe^{59} (case No. 5, Th. major splenectomized)

an elevation of counting rates of Fe^{59} on the spleen and on the sacral bone marrow with a maximum of activity on the first two days and with a constant high level for all the period of observation, a low number of counts on the liver with the maximum of accumulation at the half life of tagged red cells was the characteristic

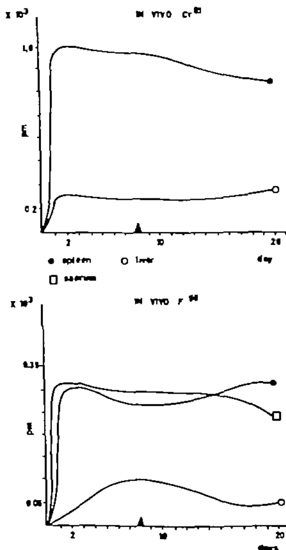


Fig 8. In vivo counting pattern over different body sites after administration of Cr^{51} and Fe^{59} (case No. 11 Th. major)

of the subjects n. 11 13 14 21 and 27 (Fig 8) The uptake of Fe^{59} by red cells may be depressed (No. 11 13 and 14) or high with a curve of haemolytic type (No 21 and 27)

In the cases No 8, 16 and 22 the utilization of Fe^{59} by red cells was of 100 % with a curve of haemolytic type, the counts over the liver and the spleen showed a rapid rise followed by a slight fall

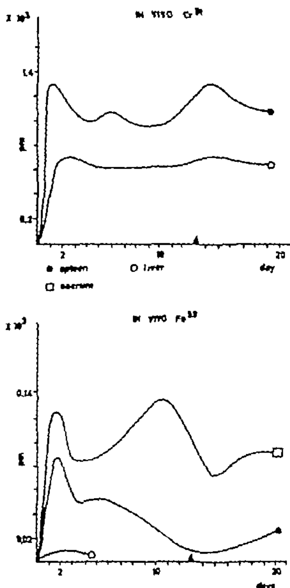


Fig. 5. In vivo counting pattern over different body sites after administration of Cr^{51} and Fe^{59} (case No. 22, Th. major).

(No. 16 after 16 days and No. 22 after 2 days when the T½ Cr^{51} was of 16 days) (Fig. 9) or multiple falls (No. 8 with the T½ Cr^{51} of 16 days). The counts over the sacral bone *marrow rose* rapidly and did not show fall after the maximum (No. 8) or they showed a pattern similar to this observed over liver and of spleen (No. 16

and 22) In these subjects the plasma radioiron clearance was not linear

In severe cases of thalassemia with clinical appearance in the first months of life (No. 7bis and 24) the clearance of radioiron was not linear the uptake of Fe^{59} by red cells was of 100 / the counts over the spleen were low over the sacral bone marrow they rose slightly and over the liver they rose progressively with a values very high (ratio liver/bone marrow 1 6) The T½ Cr^{51} of tagged red cells were in both subjects of 8 days.

In the cases No. 25 26 and 29 the plasma radioiron clearance was not linear the uptake of Fe^{59} by erythrocytes was of 100 /₀ the counts over the liver the spleen and the sacrum had a similar pattern with progressive rise in the first days and a definite plateau (No. 26 and 29) while in the case No. 26 the counts over the sacrum bone marrow were depressed The case No 26 was a child, 2 years old, with severe thalassemia while the children 25 and 29 aged 7 and 8 years, had a moderate thalassemia.

The analysis of results of the counts in *whole blood* and in *stroma-free haemolysates* carried out in the subjects No 4 5 13 and 24 showed that the activity of Fe^{59} in the haemolysates is less in the cases No. 4 13 and 24 (Fig 10)

Discussion

The shortened survival of thalassemic erythrocytes is a mechanism of the anemia in thalassemia major and confirms the presence of a haemolytic process. The different methods used for the demonstration of the survival reduction are concordant to show a marked reduction of red cells life span in thalassemia major with marked variations between several subjects. The first studies were carried out with the Ashby differential agglutination technique and show a mean cell survival of 10–19 days (1–3) The use of Cr^{51} as red cell label has confirmed these results and has permitted a large study of the survival of autotransfused labelled erythrocytes and of sites of red cells destruction in thalassemia (5 7 9 13 17 19 20) The existence of a red cells population of short life was found by Bayley and Prankerd (6) from analysis of their survival curves. They show in the curves two phases a initial rapid fall followed by a rise of surface counting over the spleen with remotion of 15–20 / of the labelled red cells within two days and a slower phase of moderately shortened survival. Vullo and Tunioli (9) have ob-

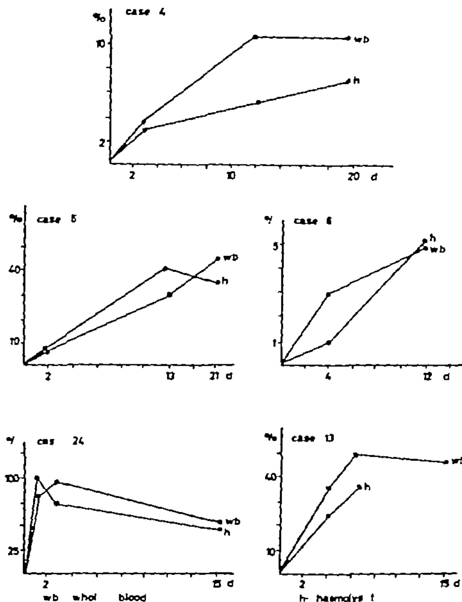


Fig. 10. Iron[59] utilization per g of haemoglobin in whole blood and in stromafree haemolysate.

served like findings transfusing thalassemic erythrocytes labelled with Cr^{51} to normal subjects. On the contrary a single component Cr^{51} survival curves has been observed by Malamos *et al.* (15). They think that the number of erythrocytes of very short life would be very small in total cell population.

The survival of the thalassemic erythrocytes has been shown strongly reduced in our experience. The production index calculated is increased till 10 times but not with constant ratio with destruction index. Consequently the compensation index is always diminished.

The study of the sites of destruction of tagged red cells shows a rise of counting rate over the spleen during the destruction of tagged red cells with high ratio spleen/liver in 18 subjects. Only in few subjects the counting rates over the spleen and liver rises simultaneously. Our findings about the surface counts are like to the findings observed (6 13 17 19 20). On the contrary MALAMOS *et al.* (13) refer that the accumulation of Cr^{51} in the spleen during the destruction of Cr^{51} labeled cells is only moderate in most patients investigated.

We believe that the destruction of thalassemic erythrocytes by spleen is of first value and increases when the subjects advance in age. The favourable effect of splenectomy in our subjects supports *this hypothesis in spite of the subsequent increase of the erythrocytes* destruction in the liver (17 19 41).

Despite of the reduction of red cell hemoglobin content in thalassemia serum iron is high and serum iron binding capacity often fully saturated and stainable iron is found in large amounts in reticulo-endotelial system (42–46). Hypochromia and the marked morphologic abnormalities of red blood cells toghether with an elevated serum iron have suggested a defect in hemoglobin synthesis in thalassemia major related to the quantitative defect in heme synthesis (10 21). Recently the inability to synthesize normal beta chains of globin has been suggested (22 23) to explain the primary defect in hemoglobin synthesis in thalassemia. The marked depression of delta Alase and of heme-synthetase activity in erythroid cells of bone marrow (27 28) and consequentely of heme synthesis pathway may be determined by a secondary phenomenon for a negative feedback mechanism.

The studies carried out by us *in vivo* on iron metabolism by $iron^{59}$ tracer has shown marked and constant alterations of iron turnover of thalassemic major children. The plasma Fe^{59} clearance curve shows a rapid disappearance of the plasma radioiron (5 6, 8, 13 14 15 18). The aspect of the most plasma Fe^{59} clearance curves suggests the possibility of a quickly re-circulation or re-utilization of radioiron that is early rapedly removed from plasma and afterwards

returns to it. The possibility demonstrated by us of a first and characteristic dip of plasma radioiron curve followed by a rise and by a second and constant dip is due probably to the return of iron to plasma from destroyed immature red cells and is probably related to the ineffective erythropoiesis. The rise of the radioactivity on the liver confirms these hypothesis. A transient elevation of plasma radioiron has been observed by GEVIRTZ *et al* (47) in thalassemia minor and by MOVITT *et al.* (48) in the sickle cell-hemoglobin C disease.

Plasma iron turnover and red cell iron turnover rate are very increased in thalassemia major (5 8, 13 14 15 18) It is remarkable that the values of plasma iron turnover rate in thalassemia may be considered over-estimated because the estimation of the plasma iron turnover rate is made in terms of a single exponential incorrected function (38, 49) due to curvilinear form of the plasma Fe^{59} clear ance curve. Red cell iron turnover is increased in the thalassemia major but it is probably over-estimated because of the absence of a clearly defined plateau in the red cell Fe^{59} uptake curve. Under this circumstance the red cell iron renewed per day results strongly increased.

The red cell Fe^{59} utilization in normal subjects represents the haemoglobin synthesis but in thalassemia subjects does not ne cessary represent it. However large deposits of non-haemoglobin iron has been demonstrated in the red cells of patiens with thalassemia major by electronic microscopy (50 51) by cythochemical studies (52) by chemical determinations (53) and by iron^{59} (10) The thalassemic reticulocyte loads in vain one third of iron that has fixed *in vitro* with an high value. This is probably the expression of a high uptake that should be referred to a primitive impairment in thalassemia and not to an alteration secondary to reduced haemoglobin synthesis (54 55 56, 24) Recently MALAMOS *et al.* (57) have demonstrated in the soluble portion of the thalassemic erythrocytes the presence of *non haemoglobin iron-bearing component.*

The property of the thalassemic reticulocyte is important for the interpretation of the utilization curves of radioiron that we have observed in our subjects. The curves of the type I and II are the expression of the reduced utilization of Fe^{59} for the haemoglobin synthesis the curves of the type III however show a paradoxal and highest utilization of the Fe^{59} with a greater rate of appearance of the radioactivity in peripheral blood. We have also investigated

in some subjects if the high radioactivity is related to non-haemoglobin Fe^{59} bound to the stroma but our results show that only a moderate activity of the iron is removed with the stroma in according to MALAMOS *et al.* (58)

We believe that the immature red cells of thalassemia major have an abnormal capacity of iron-uptake and show an abnormal fixation of the iron in the bone marrow This findings that are present in some of our subjects have not been observed in the previous researches on erythrokinetics in thalassemia. Only in the studies of GABUZDA *et al.* (59) it was referred that in one patient a large fraction of red cells rich in haemoglobin A exhibited a half life of only one day

Certain methodological problems must be considered when we estimate the data obtained from measurement of red cell production and destruction with Fe^{59} and Cr^{51} Those inherent to iron include dilution of the tracer in enlarged iron stores, recycling of iron from early destruction of tagged red cells and from stores, synthesis of iron-containing enzymes other than haemoglobin and other temporary shunts of iron from the plasma, uptake of the tracer into reticulocytes. The principal problem of the kinetic studies of Cr^{51} tagged red cells is the elution rate that must be the same for both normal and all varieties of abnormal erythrocytes (29)

Despite of these limitations it is possible that in thalassemia major the rate of erythropoiesis is moderately increased whereas red cell destruction is strongly increased. The iron metabolism is also strongly impaired but the relations between the impairment of iron metabolism and the defect of haemoglobin synthesis are not clear The use of iron^{59} is not sufficient to explain this problem *in vivo* because the results are over-estimated and it is not possible to obtain an exact quantitative measurement of the iron metabolism. In spite of it the alterations of iron metabolism in thalassemia are constant and not related to clinical status of the subject.

Summary

A double radioactive method with Fe^{59} and Cr^{51} has been used for studying the erythrokinetics in children with thalassemia major The rate of erythropoiesis is moderately increased whereas red cell destruction is strongly increased. The iron metabolism is strongly impaired and it is not clear if the impairement is primary or secondary to the defect of haemoglobin synthesis. The alterations of iron metabolism are constant and not related to the clinical picture.

Zusammenfassung

Bei Kindern mit Thalassaemia major wurde die Erythrokinese untersucht mit Hilfe einer zweifachen Isotopenmethode mit Fe^{59} und Cr^{51}. Die Erythropoese ist geringgradig, der Abbau der Erythrocyten ist stark gesteigert. Der Eisenstoffwechsel ist schwer verändert. Es ist unklar, ob diese Störung primär ist oder sekundär als Folge eines Defektes der Hämoglobinsynthese. Die Veränderungen des Eisenstoffwechsels sind konstant und stehen nicht in Beziehung zum klinischen Bild.

Résumé

L'érythrocinétique a été étudiée à l'aide d'une méthode employant du Fe^{59} et du Cr^{51} chez des enfants atteints de thalassémie majeure. L'érythropoïèse est légèrement augmentée, la dégradation des érythrocytes l'est fortement. Le métabolisme du fer est gravement perturbé, et il n'est pas certain que cette perturbation soit primaire ou vienne d'une défectuosité de la synthèse de l'hémoglobine. Les altérations du métabolisme du fer sont constantes et ne sont pas en relation avec le tableau clinique.

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Relationship of Paroxysmal Nocturnal Hemoglobinuria to Other pH Dependent Hemolytic Systems: Role of Acetylcholinesterase

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In our studies of pH and properdin-dependent hemolytic systems resembling that of paroxysmal nocturnal hemoglobinuria (PNH) measurements were made of the erythrocyte acetylcholinesterase (ACHE) The ACHE level is lower in all PNH red cells, more so in the reticulocyte. PNH red cells surviving repeated acid hemolysis have a higher enzyme titer than does whole blood prior to hemolysis. FIRKIN et al. (1) have demonstrated that incubation of human RBC with trypsin decreases the ACHE content, but does not interfere with the susceptibility of such enzyme-treated RBC to acid hemolysis. In this note we wish to extend the *comparison between the characteristics of the PNH red cell and* the pH and properdin-dependent hemolytic systems with particular reference to ACHE levels.

Materials and Methods

Bromelain, cholera vibrio extract, ficin, papain, and trypsin were used to alter the normal red cell membrane (2) ACHE levels were measured by the electrometric method of MICHEL (3) Antibody sensitized red cells were prepared by exposure of type 0 Rh positive cells to human anti-Rh 0 (Anti-D) serum. Reduced glutathione levels were measured by the method of PHILLIPS and GRUNERT as modified by BEUTLER (4) Glutathione stability tests were performed according to the method of BEUTLER (4).

Results

Of those agents used to alter the red cell membrane, bromelain ficin papain and trypsin were the most efficacious in decreasing ACHE activity (Table I) Red cells sensitized by antibody do not have a decreased ACHE level. The amount of proteolytic enzyme

Table I
Acetylcholinesterase level of red cells treated with various enzymes

Enzyme	Per cent
Bromelain	4
Ficin	8
Papain	13
Trypsin	13
Cholera vibrio extract	93

Expressed as percentage of the ACHE level remaining after treatment with enzyme (average of four determinations).

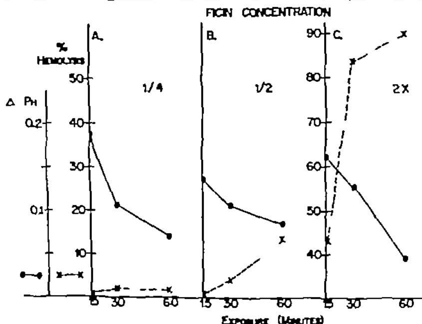


Fig 1 Ficin incubation mixture (1 pt. ficin suspension 1 pt. packed cells) *A* 6.2 mg% ficin suspension, *B* 12.5 mg% ficin suspension, *C* 50.0 mg% ficin suspension. Ficin treated RBC, which had been prepared by the above varying incubation conditions, were made into 20 percent saline suspension. 0.05 ml was added to 1.0 ml compatible serum pH 6.3. After 30 min, the degree of hemolysis was measured colorimetrically at 540 nm.

required to produce the acid hemolysis lesion of the treated RBC (HAM test) was compared to that needed to decrease ACHE activity Fig 1 depicts a typical experiment using ficin as the sensitizing enzyme. Ficin was used at ¼, ½ and two times the usual concentration. Each concentration was exposed to the red cells at ¼ ½, and one times the usual exposure time i. e., 15 30

and 60 min Less proteolytic enzyme treatment is required to produce a decrease of cholinesterase activity than is required to manifest acid hemolysis of these cells.

When red cell ACHE levels were lowered by enzymatic digestion with either bromelain, ficin, papain, or trypsin, and subsequently hemolyzed by repeated incubation in fresh acidified serum, the ACHE level of cells surviving hemolysis did not change significantly Glutathione levels were not altered by treatment with the bromelain, ficin, trypsin, or by exposure to cholera vibrio extract.

Discussion

Our data on the inactivation of ACHE activity of red cells treated with the proteolytic enzymes bromelain, papain and trypsin confirm the investigations of HERZ (5) and FIRKIN (1) In addition, we have found that the enzyme ficin is also an inactivator of ACHE. HERZ (5) showed that neuraminidase does not decrease the activity of ACHE Our results with cholera vibrio extract confirm this. As an incidental observation, we were not successful in affecting the ACHE level of red cells damaged by antibody KAPLAN (6) observed that the erythrocyte ACHE is reduced in ABO hemolytic disease of the newborn. However treatment of normal red cells with isoantibodies *in vitro* or *in vivo* by transfusion of type 0 plasma into a type B hemophiliac recipient likewise failed to lower the ACHE level.

The proteolytic enzymes act by hydrolyzing specific peptide bonds of the lipoprotein surface of the red cell membrane. NORTHROP (7) has demonstrated that trypsin does not permeate the membrane of the living cell. Intracellular enzymes such as lactic dehydrogenase, glucose-6-phosphate dehydrogenase, acid phosphatase, and inorganic pyrophosphatase are not affected by proteolytic enzymes (5) No alteration of the reduced glutathione level or of glutathione stability was observed in cells treated with *bromelain, ficin or trypsin. Most observers believe ACHE is located* in the stromal fraction of the red cell. Because red cells treated with proteolytic enzymes are susceptible to acid hemolysis, we compared the amount of enzyme sensitization necessary to produce acid hemolysis and to decrease cholinesterase activity A relatively small exposure to the proteolytic enzyme will cause the ACHE activity to fall before an appreciable degree of acid hemolysis

appears (Fig 1) This is in substantial agreement with the work of PERONA (8) The observation would be consistent with a deeper (or less accessible) site for the acid hemolysis lesion. When a sample of normal blood is separated by centrifugal methods into young and old populations which are then identically treated with proteolytic enzymes, the old cells undergo greater hemolysis than do the young cells* Because the ACHE level is higher in young cells (9 10 11) one might expect the enzyme-treated cells surviving repeated acid hemolysis to have a higher ACHE level. However no significant change was detected in the ACHE level of enzyme-treated cells after repeated acid hemolysis. This is in contra distinction to the work of METZ (12) with PNH, in which those cells that survived repeated acid hemolysis are higher in ACHE. That loss of ACHE is not of itself lethal to the red cell was shown by METZ (13) He demonstrated that despite *in vivo* irreversible inhibition of RBC ACHE there was no effect on the erythrocyte lifespan. Moreover JOHNS (14) has reported a case of familial re duction in red cell ACHE in an apparently healthy man. By contrast, those cells treated with proteolytic enzymes shown a marked reduction in lifespan (15)** Although the ACHE level and results of the Ham test can be correlated with the shortened survival of PNH cells and the clinical severity of the disease, it appears highly unlikely that any direct association exists between ACHE activity and the structural integrity of the red cell.

Summary

Treatment of red cells by various proteolytic enzymes is known to produce pH and properdin-dependent hemolytic system similar to that of PNH. In the red cells of this disease, as well as in the enzyme-treated cells, ACHE level is low Of the enzymes studied the most efficacious in reducing the ACHE level were bromelain, ficin, papain, and trypsin. Less treatment with proteolytic enzyme is required to produce decrease in ACHE activity than is required to demonstrate acid hemolysis. When enzyme treated cells are subjected to repeated hemolysis in fresh acidified serum, there is no preferential hemolysis of those cells with lower ACHE level. The results are discussed in relation to certain known characteristics of the PNH red cell.

Zusammenfassung

Die Behandlung von Erythrocyten mit verschiedenen proteolytischen Enzymen erzeugt ein pH und Properdin-abhängiges hämolytisches System ähnlich demjenigen der PNH. Die Erythrocyten dieser Krankheit, sowie auch Enzym-behandelter Zellen

* Unpublished data (LAPOSKY).
** Unpublished data (LAPOSKY and KA)

weisen einen niedrigen Gehalt an Acetylcholinesterase (ACHE) auf. Von den untersuchten Enzymen waren mit Bezug auf die Reduktion der ACHE Bromelain, Ficin, Papain und Trypsin am wirksamsten. Zur Herabsetzung der ACHE-Aktivität ist eine geringere Behandlung mit proteolytischem Enzym erforderlich als zum Nachweis der Säurehämolyse. Wenn Enzym-behandelte Zellen einer wiederholten Hämolyse in frischem angesäuertem Serum unterworfen werden, findet sich keine stärkere Hämolyse der Zellen mit niedrigem Gehalt an ACHE. Die Resultate werden im Hinblick auf gewisse bekannte Eigenschaften der PNH Erythrocyten diskutiert.

Résumé

Il est connu que le traitement d'érythrocytes par différents enzymes produit un système hémolytique dépendant du pH et de la properdine, système semblable à celui de l'hémoglobinurie nocturne paroxystique (HNP) Le taux d' cétylcholin-estérase (ACHE) est aussi bas dans les érythrocytes de cette maladie que dans les cellules traitées par des ferments. Parmi les enzymes étudiés, les plus efficaces à diminuer le taux d'ACHE étaient la bromelaine, la ficine, la papaïne et la trypsine. Le traitement à l'aide de ferments protéolytiques n pas besoin d'être aussi intense pour démontrer une hémolyse par acidification que pour diminuer l'activité de l ACHE. Quand les *cellules traitées aux ferments sont soumises à une hémolyse répétée dans du sérum* fraîchement acidifié, l'hémolyse n est pas plus marquée parmi les cellules ayant un taux en ACHE bas. Les résultats sont discutés en relation avec certaines caractéristiques connues des érythrocytes de l'HNP

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Survey for Erythrocyte Glucose 6 Phosphate Dehydrogenase Deficiency in Hawaii*

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Erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is prevalent amongst certain ethnic groups. Ethnic groups showing a high incidence for G-6-PD deficiency include American and African Negroes, Caucasians of the Mediterranean, Middle and Near East regions, certain Asiatic groups, Mexicans and Oyana Indians in Surinam of South America (1 2 3) In Hawaii the multi-racial population presents a unique opportunity to study the various ethnic groups for G-6-PD deficiency This report summarizes a search for G-6-PD deficiency amongst the many ethnic groups living in Hawaii.

Materials and Methods

G-6-PD activity was determined on freshly drawn oxalated blood by the qualitative method of Motulsky and Campbell (4) The decolorization time for G-6-PD activity in normal individuals ranged from 1 to 2 h. Blood specimens were obtained from normal individuals, from healthy blood donors at the Blood Bank of Hawaii and from patients with variety of haematologic and non-haematologic diseases, irrespective of sex. Low hematocrit blood specimens were corrected from anaemia by the removal of sufficient plasma to produce normal hematocrit levels.

Results

G-6-PD determinations were performed on 754 individuals. 196 determinations were done on normal individuals and 558 determinations on patients with various diseases** G-6-PD deficiency

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** The ethnic backgrounds for these individuals included 173 Japanese, 156 Chinese, 119 Filipinos, 105 Caucasians, 34 Portuguese, 27 Hawaiians, 76 part Hawaiians and 63 other mixed or miscellaneous ethnic groups.

Table I
Individuals with G-6-PD deficiency

Clinical diagnosis	Decolorization time—hours	Age	Sex	Ethnic composition
(A) Complete G-6-PD deficiency				
(1) Neo-natal hyperbilirubinaemia (DC)	0	newborn	M	Chinese
(2) Normal individual	0	35	M	Filipino
(3) Haemoglobin H disease	0	2	M	Filipino
(4) Haemoglobin H disease	0	56	M	Filipino
(5) Normal individual	0	48	M	Chinese
(6) Thalassaemia minor plus post-partum pregnancy	0	30	F	Filipino
(B) Partial G-6-PD deficiency				
(1) Normal individual (maternal aunt)	2 h 5'	38	F	Chinese
(2) Normal individual (maternal grandmother)	2½	60	F	Chinese
(3) Normal individual (maternal aunt)	3	28	F	Chinese
(4) Iron deficiency anaemia	3	40	F	Chinese Filipino-Spanish
(5) Normal individual (cousin)	3 h 10'	2 months	F	Chinese
(6) Normal individual (cousin)	3 h 10'	14	F	Chinese
(7) Normal individual (cousin)	3½	11	F	Chinese
(8) Normal individual* (cousin)	4	5	F	Chinese
(9) Idiopathic, acute acquired haemolytic anaemia	4	55	M	Filipino
(10) Normal individual	6	32	M	Chinese
(11) Normal individual (mother of No. 13)	7	65	F	Chinese
(12) Normal individual	8	21	M	Chinese
(13) Congenital non-spherocytic haemolytic anaemia	8	27	M	Chinese-Hawaiian
(14) Normal individual (maternal uncle)	11	41	M	Chinese
(15) Normal individual	11	35	M	Filipino
(16) Normal individual (maternal uncle)	20	31	M	Chinese
(17) Normal individual	24	56	M	Filipino
(18) Normal individual	24	62	M	Filipino

Relatives of DC (this individual has been published as case report—see reference 18)
G-6-PD activity also determined quantitatively—81 units/100 ml of RBC were found in individual No. 11 and 26 units/100 ml of RBC were found in individual No. 13 (method of ZINKHAM, normal range 150 to 215 units/100 ml of RBC)
* Niece of this individual died at the age of 18 of acute fulminating aplastic anaemia.

Table II

Gene Frequencies in Chinese, Filipinos and their Hybrids. The gene frequency in this sample tends to be higher than in the general population because of disproportionate representation of hematology patients and their relatives. Including these gives an upper limit to the gene frequency estimate excluding them gives lower limit. The upper estimate is obtained by dividing affected males by total males (e. g. /₇₀ for Filipinos) The lower estimate is obtained by excluding patients and relatives (e. g., /₇₀ for Filipinos) The gene frequency in part-Chinese and part Filipino hybrids is less than half the frequency in the pure races. While not statistically significant, this is to be expected both because the proportion of Chinese and Filipino ancestry is less than 50% in many of these hybrids, and because Filipino males outcross in Hawaii more frequently than the smaller group of Filipino females.

Race	Total males	Affected males excluding patients and relatives	Total	Gene frequency
Chinese	86	3	6	0.036—0.070
Filipino	70	4	7	0.060—0.100
Hybrids	63	0	1	0—0.016

was found in 24 individuals. In 6 individuals the G-6-PD deficiency was complete, i. e. no decolorization occurred. In 18 individuals the G-6-PD deficiency partial, as evidenced by decolorization times ranging from 2 to 24 h. The clinical data, ethnic backgrounds and decolorization times for these 24 individuals are listed in Table I. The gene frequencies for those individuals in whom G-6-PD deficiency were found are listed in Table II

Discussion

G-6-PD deficiency was found primarily in individuals of Chinese or Filipino ethnic backgrounds in Hawaii. These two ethnic groups also exhibit a high incidence for thalassaemia and haemoglobin H disease (5) *G-6-PD deficiency as well as thalassaemia* and haemoglobin H disease have been observed in Chinese and Filipinos in other parts of the world (3 6, 7) G-6-PD deficiency is also prevalent in other Far Eastern ethnic groups, including Thais, Malayans, Indians, Melaneasians in New Guinea and New Britain, certain Micronesians, and Indonesians (3 6, 7 8, 9) In certain oriental racial groups, primarily the Japanese, thalassaemia and abnormal haemoglobinopathies occur less commonly While isolated cases of thalassaemia and abnormal haemoglobins have been found, mention has been made of the absence of G-6-PD deficiency in Japan (3) Neither G-6-PD deficiency thalassaemia or

abnormal haemoglobins have been encountered in the Japanese in Hawaii to date. Ethnic groups surrounding the Mediterranean, Middle and Near East regions, i. e., the Italians, Greeks, Sicilians, Sardinians, Sephardic Jews, Iranians, Persians, Turks, Saudi Arabians, certain Dutch and Amboynenans in The Netherlands also showed a high incidence for G-6-PD deficiency (1 10 11) Thalassaemia, haemoglobin H disease and G-6-PD deficiency appear to occur commonly amongst certain ethnic groups of the Mediterranean, Middle and Near East and Asiatic regions. Two of these three defects have occurred in the same individual, i e. G-6-PD deficiency combined with thalassaemia (12) and G-6-PD deficiency associated with haemoglobin H disease (13 14)

The incidence for G-6-PD deficiency amongst the Chinese and Filipinos in Hawaii are 8.9 and 6 7% respectively In Singapore Vella has found the incidence for G-6-PD deficiency in healthy Chinese adult males blood donors to be 2.5% (15) Chan *et al.* has found 5.5% of adult Chinese males from the southern China province of Kwantung to be deficient in G-6-PD activity (16) Lee *et al.* have found an incidence of G-6-PD deficiency ranging from 0.33 to 6.25 % amongst the Chinese of both sexes in Taiwan (17) In Taiwan Chinese migrants from the Fuchien Province in China demonstrated the lowest whereas the Hakka Chinese migrants from the Kwantung Province in China demonstrated the highest incidence for G-6-PD deficiency The majority of Chinese in Hawaii have migrated from the Canton, Hakka, Hiang Shan (Chung Shan) cities and villages within the Kwantung Province. The apparent higher incidence of G-6-PD deficiency in Chinese within the Kwantung Province region may be related to the observation that malaria existed in these areas until very recently (17) In the Phillippines Buenaventura has found approximately 6 % of Filipinos to be deficient in G-6-PD activity (7)

Acknowledgement. Appreciation is expressed to Newton E. Morton, Ph. D Chairman and Professor of the Department of Genetics, University of Hawaii, who performed the gene frequency calculations.

Summary

A survey for G-6-PD deficiency was carried out amongst the multi-racial population in Hawaii. Of 734 individuals investigated, 24 were found to have G-6-PD deficiency Six individuals were found to have complete, and 18, partial G-6-PD defi-

ciency The G-6-PD deficient individuals were of primarily Chinese or Filipino ethnic extraction. The overall incidence for G-6-PD deficiency amongst the Chinese and Filipinos for both sexes in Hawaii is 8.9 and 6.7% respectively

Zusammenfassung

In der vielrassigen Bevölkerung von Hawaii wurde eine Untersuchung über die Häufigkeit des Mangels an Glukose-6-Phosphatdehydrogenase (G-6-PD) durchgeführt. Von 754 untersuchten Individuen zeigten 24 einen G-6-PD-Mangel. Der Mangel war bei 12 Individuen komplett, bei 18 partiell. Die Individuen mit G-6-PD-Mangel waren primär chinesischer oder philippinischer Abstammung. Die gesamte Häufigkeit des G-6-PD-Mangels unter Chinesen und Philippinen beiderlei Geschlechts in Hawaii beträgt 8,9 bzw 6,7%

Résumé

Une étude sur la fréquence du manque de G-6-PD été faite parmi la population multiraciale de Hawaï. Parmi 754 individus examinés, 24 avaient un manque de G-6-PD. Chez 6 individus, il était total, chez 18 partiel. Les individus ayant un manque en G-6-PD étaient à l'origine de souche chinoise ou philippine. La fréquence totale du manque de G-6-PD parmi les Chinois et les Philippins des deux sexes était à Hawaï de 8,9% respectivement de 6,7%

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The Potential of Lymphocytes from Patients with Leukaemia and Reticuloses to Transform under the Influence of Phytohaemagglutinin

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A number of reports have appeared recently concerning the ability of the small lymphocyte from patients with chronic lymphatic leukaemia to transform and undergo *mitosis under the influence* of phytohaemagglutinin. In an earlier report we described such a normal degree of transformation (7) and more recently we have also described a low degree of transformation in a group of patients with this disease who had the complication of hypogamma globulinaemia (8) Others have regularly found a decreased degree of transformation in cultures of lymphocytes from patients with this type of leukaemia (1 2, 3 16 19 21) In this paper we describe the transformation of lymphocytes from a group of patients with chronic lymphatic leukaemia, and also the effect of normal plasma on these cells, and of leukaemic plasma on normal cells. Similar studies have also been made using cells and plasma from patients with Hodgkin s disease.

Methods

Leucocytes were obtained from whole blood by the dextran sedimentation method and the plasma removed from them by centrifugation t 1000 rpm for 20 min. The cells were resuspended in volume of culture medium 199 equal to the amount of plasma removed and cell count was made. Cultures were then set up which contained approx*imately 1000 cells per mm³ and either normal or leukaemic plasma was added to give* final concentration of 20%. Normal cells were set up with their own plasma, with leukaemic plasma, and with plasma from patients with Hodgkin's disease. Lymphocytes from patients with chronic lymphatic leukaemia were set up in cultures containing either autologous plasma or with plasma from least 3 normal subjects cells from subjects with Hodgkin s disease were treated in the same way Phytohaemagglutinin (P) was added to each culture in concentration of 0.05 ml/10 ml of culture. The same batch of PHA was used throughout this work. Cultures were terminated after 72 h of incubation at 37°C and smears of the cells made and stained with Jenner-Giemsa stain combination.

Subjects

36 healthy volunteers were used as donors of normal lymphocytes. 24 patients with chronic lymphatic leukaemia were studied; all had the typical blood, marrow and clinical features of the disease, although one case was in the terminal leukaemic phase of lymphoid reticulosis (No. 17) Only one patient had clinical evidence of hypogammaglobulinaemia (No. 6), although low globulin levels were found in another four patients which, on electrophoresis, were found to be due to deficiency in the gammaglobulin fraction. None of our previously reported hypogammaglobulinaemic subjects were included in this series. Five patients with Hodgkin disease were studied, and some cultures of normal cells were treated with plasma from five patients with chronic myeloid leukaemia, two of whom were in sub-acute phase of the disease, and one patient with acute myeloblastic leukaemia.

Results

The degree of blast transformation, together with the total lymphocyte counts and serum globulin estimations, are shown in Table I From this data it will be seen that only seven of the leukaemic cell cultures, in their own plasma, had a percentage of blasts which was within the normal range. A further seven cultures contained more than 20 / blasts and the remainder showed very poor degrees of transformation. From this table it will be seen that culturing the leukaemic cells in the presence of normal plasma had little modifying effect.

The degree of transformation found in cultures of lymphocytes from patients with Hodgkin s disease are shown in Table II, from which it will be seen that the percentage of blasts in all cultures with autologous plasma was within normal limits and was not influenced by the presence of normal plasma.

The data obtained from the normal subjects are summarised in Table III The presence of neither leukaemic plasma nor that from patients with Hodgkin s disease affected the percentage of blasts in culture. The results of normal cell cultures in the presence of plasma from patients with myeloid leukaemia are shown separately in Table IV From this it will be seen that in general these plasma have little effect on the cell transformation. In four cultures there was a reduction in the percentage of blasts however and in these cultures the cells were in an unhealthy state and much lysis was in evidence.

Discussion

This series of experiments confirms those previous findings mentioned above. Only three of our leukaemic patients have been

Table I
The transformation of lymphocytes from patients with lymphatic leukaemia.

Case No.	Peripheral blood count $(\times 10^9)/mm^3$	Serum globulin *g/100 ml*	Percent of Transformation in own plasma	normal plasma	range
1	145.0	1 4	9	10	6—14
2	4.85	1.7	56	47	47
3	19.7	—	4	—	—
4	20.3	2.5	16	—	—
5	14 4	—	9	—	—
6	42.6	1.5	14	—	—
7	8.750	3.3	6	—	—
8	47.8	—	6	7.6	5—10
9	20.3	2.9	22	26.6	21—30
10	5.10	2.5	70	73.0	72—74
11	30.6	2.2	38	39.0	36—44
12	3.7	2.2	78	76.3	75—78
13	17.6	1.9	28	20	14—26
14	39.0	2.4	48	46.5	4 —54
15	12.0	2.2	26	18.6	14—22
16	88.4	2.8	36	30.6	28—34
17	1.50	5.0	80	76	69—86
18	32.5	—	5	8.3	6—10
19	14.5	2.5	10	10	8—12
20	72.0	2.3	26	24.3	20—29
21	9.3	2.7	3	4.3	2—6
22	48.6	2.9	55	53	45—59
23	18.7	2.7	70	63	59—72
24	3.9	1.5	65	—	—

Table II
Transformation of lymphocytes from patients with Hodgkin disease.

Case No.	Percent transformation own plasma	Percent transformation normal plasma
1	70	78.6 (78—80) range
2	67	66.0 (64—70) range
3	62	59 6 (58—62) range
4	56	—
5	62	—

studied previously between two and three years ago and in two (No. 10 and 24) a normal degree of transformation was observed then and now the third (No 5) when studied previously had a

Table III

Effect of leukaemic and Hodgkin's plasma on normal cell transformation.

Normal culture			Normal cells and Hodgkin plasma			Normal cells and lymphatic leukaemia in plasma		
% (mean)	Range %	No. of subjects	% (mean)	Range %	No. of subjects	% (mean)	Range %	No. of subjects
70	45—86	36	74	64—80	9	67	47—81	66

Table IV

Effect of plasma from myeloid leukaemia on transformation of normal cells.

Case and diagnosis	Normal cultures (normal plasma) Mean %	Normal cells and CML plasma normal 1	normal 2	normal 3	normal 4	Treatment of patient
1 CML	67	74	22	2	—	T M
2. CML	63	64	0	50	—	T M
3 CML	72	76	24	47	—	T M
4. CML	70	74	64	75	63	M
5. CML	70	70	80	66	60	M
6. AML	67	85	70	71	—	M

CML chronic myeloid leukaemia
AML acute myeloid leukaemia
T transfusion
M cytotoxic

normal degree of activity but now shows a reduced number of blasts. It has been suggested that it is the plasma or serum component of the culture medium which is the causal agent in low degree of transformation in the lymphatic leukaemias (2) We have been unable to find any evidence to support such a hypothesis normal plasma will not correct the fault in the leukaemic cell cultures, and neither will leukaemic plasma inhibit transformation of normal cells.

The results of our experiments tend to point to a defect in the lymphocytes. It was considered that this defect may arise as a result of therapy with cytotoxic drugs or irradiation which may cause damage to the cells (21) The only patient in this latter series who showed substantial numbers of blast cells in culture had not received any treatment for many months. In our series only three of the patients were not receiving anti-leukaemic therapy at

the time of study and had in fact never had such treatment. Only one of these three patients had a degree of transformation within normal limits one was slightly reduced and the third was well below normal. Those others who transformed to the normal degree had received nitrogen mustard therapy for some months before study In another study in one case cultures made before treatment showed minimal transformation, but after X irradiation the total lymphocyte count on the peripheral blood fell considerably and in post treatment cultures normal numbers of blasts were present (1) Thus treatment probably has little direct role in bringing about the poor *in vitro* response of the leukaemic lymphocyte.

A consideration of the effect of treatment does however point to a possible significant reason for the behaviour of the leukaemic cells. In our series we have noticed that five of the good responders had total lymphocyte counts of less than 18000 whereas all of the 'poor responders had high total lymphocyte counts. Such a correlation with lymphocyte content of the peripheral blood has been noted previous (3 16) One possible explanation for these findings may lie in the recent studies of lymphocyte life-span. In rodents it is now well known that two populations of lymphocytes exist with respect to life span a short lived, rapidly produced population whose life-span may be up to two weeks, and a long-lived population whose life-span is probably measured in terms of months or years and are produced at about the same rate as body growth (9 14) There is some evidence from radiotracer studies (18) and the presence of radiation-induced unstable chromosome abnormalities in lymphocytes many years after the irradiation (6, 15) that such a situation probably also exists in man. There is some evidence also that it is the long-lived population which is responsible for immunological activity as this forms the bulk of the lymphocyte population of the lymph nodes and thoracic duct, and has also been found to show some changes after antigenic stimulation *in vivo* (9) The question arises as to which type of lymphocyte is abnormal in chronic lymphatic leukaemia. If the PHA transformation represents a non-specific reaction *in vitro* of the type suggested by Coulson and Chalmers (5) then it is conceivable that the low degree of transformation in some cases is due to overgrowth of the short lived population. This would result in a very small number of the long lived cells being represented in the ten million cells which are cultured. Hamilton (10 11) found two populations of

lymphocytes in lymphatic leukaemia one long lived with a life span of more than 300 days, and the other a shorter lived population with a life-span of 85 days. This finding would favour an increased longevity of the short-lived population which, if produced at the normal rate would lead to increases in their number. Other studies of the life-span of the lymphocyte which have been carried out on human chronic lymphatic leukaemia indicate that the overall life span of the lymphocyte population is increased (4, 17). Such data may suggest over-growth of the long lived population which should result in a normal or increased degree of transformation *in vitro*. If this latter situation is true then a defect in the long lived cell must exist, and such a possible defect has been suggested (19). It was found that leukaemic lymphocytes failed to show the increase in PAS activity normally seen in normal cultures with PHA and they also showed an absence of increased dehydrogenase activity which again is a feature of normal transforming lymphocytes. From these findings it was suggested that the leukaemic lymphocyte fails to elaborate the enzymes necessary for glycolysis, with subsequent failure to synthesise and utilise glycogen and, finally a failure to synthesise DNA. It must be pointed out however that the nontransforming lymphocytes in normal cultures also fail to show increases in glycogen and dehydrogenase activity. These may represent the short lived population.

Light can only be shed upon these problems by means of detailed *in vivo* studies of lymphocyte proliferation in chronic lymphatic leukaemia.

The hypogammaglobulinaemia found in some of our patients may be the result of the destruction of the immune population of lymphocytes either due to therapy or to crowding out of these cells by the leukaemic cells infiltrating the lymphoid organs. Patient No. 1 in this series had had a normal globulin level for most of the course of his disease and only in recent months has his globulin level fallen. Primary hypogammaglobulinaemia is however not always associated with failure of lymphocyte transformation and there seems to be a wide spectrum some cases show poor lymphocyte transformation (8) whereas others show normal transformation (12, 13). It is also of interest that the patient in our series showing the highest percentage of blasts also had hyperglobulinaemia this patient's disease started in his lymphoid tissue as a reticulosis and may represent overgrowth of the long lifed population. One patient

(No 6) who showed poor transformation and was found to have deficient gammaglobulin has since developed clinical signs of this deficiency.

Some comment is required concerning the effect of plasma from myeloid leukaemia on normal cells. Most of the tests showed no significant effect. Plasma from three patients however caused a reduction in the percentage of blast cells which was associated with a very poor state of the cultures: many of the cells were in the process of lysis and others were pyknotic. Each of these patients from whom the plasma had been obtained had received multiple transfusions and it is therefore possible that the deleterious effect on these cultures, and not on others, was due to leucocyte antibodies.

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Summary

A study has been made of the ability of lymphocytes from 24 patients with chronic lymphatic leukaemia to respond to stimulation with PHA *in vitro*. Normal degree of transformation was found in cultures from only seven of these patients. It was found that the degree of transformation of leukaemic cells could not be influenced by the presence of normal plasma, and chronic lymphatic leukaemia plasma had no depressant effect on the degree of transformation in normal cultures. Normal transformation was found in patients with Hodgkin's disease, and plasma from patients with Hodgkin's disease had no influence on the transformation of normal lymphocytes. In only three cultures of normal cells in the presence of plasma from patients with chronic myeloid leukaemia was any disturbance in normal transformation found. Possible explanations of the implications of these findings are discussed.

Zusammenfassung

Die Reaktion der Lymphocyten von 24 Patienten mit chronischer lymphatischer Leukämie auf die Stimulation mit Phytohämagglutinin *in vitro* wurde untersucht. Eine normale Umwandlung fand sich in Kulturen von nur 7 dieser Patienten. Das Ausmaß der Umwandlung leukämischer Zellen konnte durch normales Plasma nicht beeinflußt werden, und Plasma der chronischen lymphatischen Leukämie hatte keine hemmende Wirkung auf die Umwandlung in normalen Kulturen. Eine normale Umwandlung fand sich bei Patienten mit Hodgkinscher Krankheit, und Plasma von Patienten mit Hodgkinscher Krankheit hatte keinen Einfluß auf die Umwandlung normaler Lymphocyten. Nur in 3 Kulturen normaler Zellen führte Plasma von Patienten mit chronischer myeloischer *Leukämie zu einer Störung der normalen Umwandlung*. Die möglichen Erklärungen dieser Ergebnisse werden besprochen.

Résumé

Des lymphocytes provenant de 24 malades atteints de leucémie lymphatique chronique ont été stimulés in vitro à l'aide de phytohémagglutinine, et leurs réactions ont été étudiées. Une transformation eut lieu dans les cultures de lymphocytes de sept de ces malades. Le plasma normal resta sans influence sur le degré de transformation des cellules leucémiques, et le plasma provenant de malades atteints de leucémie lymphatique chronique n'eut aucune influence sur la transformation de cellules cultivées normales. Une transformation normale des lymphocytes provenant de malades atteints de la maladie de Hodgkin eut lieu, et le plasma de tels malades n'influença pas la transformation de lymphocytes normaux. Le plasma de malades atteints de leucémie myéloïde chronique ne provoqua des perturbations que dans trois cultures de cellules normales. Les explications possibles de ces résultats sont discutées.

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Failure of Shielding the Thymus to Induce Recovery of Bone Marrow after Radiation

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Although the function of the thymus in the newborn animal in relation to the lymphatic system and the development of the immunological reaction is now well established (17) there is still doubt as to the function of this gland in the adult. In some animals the turnover of cells in the young adult is very high, but the eventual fate of the cells produced is uncertain. It has been shown that an intact thymus is essential in the recovery of the immune mechanism in the irradiated adult mouse (3) and the transplantation of the thymus hastens the regeneration of lymphatic tissues in the mouse after irradiation (8). It has also been shown in the mouse that bone marrow cells can repopulate the thymus after irradiation (16).

The present study was undertaken to determine whether or not the thymus had any potential to repopulate the bone marrow after irradiation. A lethal dose of irradiation was used in the study in order to provide the maximal stimulus for this to occur while at the same time excluding the problems of immune reaction and transplantation difficulties, by shielding the thymus of the animals during irradiation.

Materials and Methods

Guinea pigs 200 to 400 g in weight were used. Irradiation was carried out with a cobalt 60 source at a target distance of 90 cm. The dose used was 400 r which according to Ellinger (4) is a lethal dose for the guinea pig. No precautions were taken to ensure uniform dosage over the whole body of the animal. For shielding, a block of lead 10 cm in length and in cross section 4 × 2 cm was used, placed 6 cm above the animal.

In the first series the animals were divided into two groups, experimental and control, with 8 animals in each group. The experimental group had the neck region containing the thymus shielded during irradiation, while the control group were totally irradiated. One animal from each group was sacrificed at 18 h, 3 days, 5 days, 7 days, 10 days and 1[illegible] days. None of the control animals lived longer than 12 days after irradia-

tion, but the remaining animals of the experimental group were sacrified t 14 days. I order to compare cellular proliferation rates between the experimental animals and control, tritiated thymidine (specific activity 6.05 [mM) t dosage of 1 μc/g was administered to each animal 24 h prior to sacrifice except in th case of the animals sacrificed t 18 h when it was given just after irradiation.

I the second series 7 animals were thymectomised tw weeks prior to irradiation with the neck region shielded. One animal was sacrificed at each of the following intervals, 24 h, 3 days, 7 days, 8 days, nd three animals at 21 days after being irradiated. Three other animals were thymectomised and totally irradiated. One of these was sacrificed t 3 days, another t 8 day and the third died on the 9th day post irradiation. Except for the last animal tritiated thymidine was administered 24 h before sacrifice as in the first series of animals.

For the third series 12 animals were divided into experimental and control groups. Prior to irradiation the animals were anaesthetised and both lobes of the thymus were withdrawn through an incision in the skin of the neck, taking great care to maintain the blood supply to the gland. The procedure for irradiating each animal was to place it on its side with the thymus pulled away from the neck on vascula stalk (Fig 1). By this means it was possible to shield the thymus, and only the thymus, in the experimental group, while the control group were totally irradiated as before. The thymus was replaced after irradiation and the skin sutured. The animals were sacrificed in pairs, one experimental and one control t the following intervals, 18 h 24 h, 3 days, 5 days, 7 days and 9 days. Tritiated thymidine at 1 μc/g body weight for this series was given 3 h prior to sacrifice.

In all three series of experiments, samples of thymus, spleen, mesenteric node and the femoral bone marrow were removed and smears were made. Radioautographs were prepared from these smears using NTB 3 emulsion (Eastman Kodak). Other samples of these tissues were taken for liquid scintillation measurements and were also analysed for DNA content using the method of CERIOTTI (2).

Only the results from the analysis of the bone marrow and thymus are reported here.

Results

First series For the first seven days after irradiation there was little difference in the general appearance of the guinea pigs. They appeared healthy and after a loss on the day following irradiation their weights began to increase again. After this 7 day period the control animals appeared to become anaemic and the exposed skin was pale the fur lost its gloss and the animals lost weight. As already stated none survived beyond 12 days. On the other hand the shielded animals gained weight up to the time of sacrifice.

The thymuses of the control animals showed a marked loss in weight compared with the experimental animals (Table I) though the percentage of cells taking up the radioactive thymidine was approximately the same. The amount of radioactive material in the gland per mg of tissue was higher in all the shielded animals except at 7 days, when the value was slightly below that of the control animal.



Fig. 1. Guinea pig. The thymus has been withdrawn for shielding during irradiation. Note the vascular stalk to maintain the blood supply.

Table 1
First series.

Post irradiation	Thymic weights, mg		Radioactivity cpm/mg tissue	
	shielded	control	shielded	control
18 h	499	277	1568	1436
3 days	438	176	2074	1282
5 days	507	89	3410	1432
7 days	497	150	1970	1995
10 days	474	124	1471	1278
12 days	188	81	3786	1707
14 days	312		2158	
	380		2315	

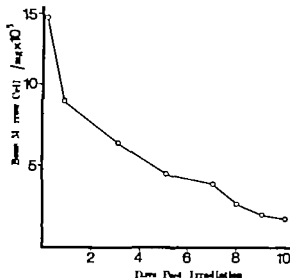


Fig 2. Graph showing the total cellularity of the femoral bone marrow after total body irradiation, based on DNA measurements.

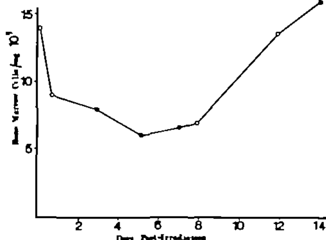


Fig 3. Graph showing the total cellularity of the femoral bone marrow after irradiation with neck shielded, based on DNA measurements.

From a determination of DNA in the bone marrow an estimate of the number of cells per mg was made assuming the DNA content per cell to be constant (Fig 2 and 3) An analysis of the types of cells present in the smears was made placing each cell in so far as possible, into one of the following four groups (1) erythroid (2) granuloid, (3) lymphoid (this group included lymphocytes, plasma

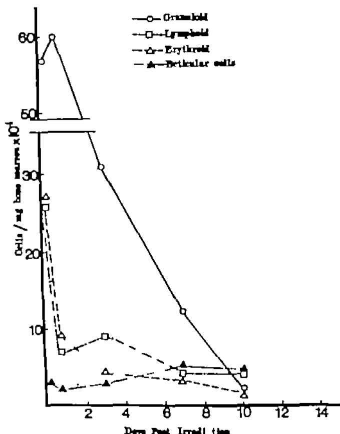


Fig 4 The numbers of cells of each of 4 groups in the femoral bone marrow are shown for totally irradiated guinea pigs.

cells, monocytes and their precursors) (4) reticular cells (large cells with a diffuse edge to the cytoplasm and lightly staining finely granular nucleus)

From the differential cell counts on the smears it was possible to estimate the actual numbers of each type using the information from DNA analysis. The results are shown in the graphs (Fig 4 and 5) It can be seen that the initial drop in the number of cells was comparable for the two groups of animals. After five days, however the recovery of the shielded animals was apparent, although the pattern of damage and recovery varied between the different types of cell. The granulocytic precursors took longer to reach their minimum numbers than either the erythroid or the lymphoid types, while the recovery of the latter two types occurred earlier than in the granulocytic cells. The reticular cells here ap-

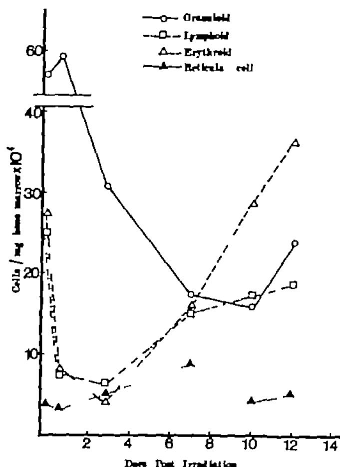


Fig. 5. Numbers of cells of each of 4 groups in the femoral bone marrow are shown for guinea pigs irradiated with the neck shielded.

peared to be radio-resistant and did not appear at any stage to be taking up thymidine to any extent.

Second series. The thymectomy wounds all healed well and none appeared infected. In this series the experimental animals reacted to the irradiation as did the experimental animals of the first series as regards their weight and appearance. The three animals which were not sacrificed until three weeks after irradiation were still gaining in weight and had all the appearances of being normal. The totally irradiated animals in general appearance were similar to the control animals in the first series. After 5 days they began to lose weight and by the eighth day their exposed skin was pale the fur had become dull and the animals were lethargic.

Table II
Third series.

Post Irradiation	Thymus weights, mg		Thymus cells/mg × 10⁶	
	shielded	control	shielded	control
18 h	226	207	2.3	1.4
24 h	301	257	2.0	1.2
3 days	270	101	2.2	.8
5 days	252	86	1.9	1.1
7 days	340	178	2.9	.9
10 days	294	96	1.8	1.1

An examination of the bone marrow in the shielded animals revealed a recovery pattern similar to that shown in the first series and in fact the points plotted for cells per mg and the various types of cells, fell on or near the curves drawn from the data of the first series. The response of the bone marrow of the non-shielded animals was comparable to that of the non-shielded animals of the first series and showed no evidence of recovery

Third series There was no gross difference in appearance between the two groups in this series after irradiation. One animal which had been shielded died on the 9th day after irradiation. They all showed a weight loss pattern similar to the control animals of the first series.

A striking feature at sacrifice was the healthy appearance of the shielded thymus, and a comparison of the weights of the thymuses in the two groups is shown in Table II Histologically there were far fewer pyknotic cells and the number of cells per unit weight of tissue was greater in the shielded animals (Table II)

The bone marrow showed no evidence of incipient recovery and in fact showed no differences from the bone marrow in the non-shielded animals. A differential analysis of the cells revealed no difference in the pattern of radiation damage in the two groups.

Discussion

ELLINGER's (4) figure of 400 r as an absolute lethal dose of irradiation for the guinea pig has been confirmed. Deaths due to irradiation occurred in the 9 to 12 days after exposure, which is in accord with the work of PHILLIPS *et al.* (22) who used X irradiation and reported that fatalities occurred in guinea pigs between 9 and

16 days after irradiation. They attributed the cause of death to haemorrhages and stated that gastro-intestinal damage was slight. Autopsies in the present study revealed numerous haemorrhages *in the spleen and intestine* and it may be that a lack of platelets combined with a reduced red cell count was the prime cause of death in these animals. It has been shown by LAW and MOLE (16) that the apparent abscopal effect on the thymus of irradiation of the abdomen in the rat was due mainly to a reduction in the food intake. In the present experiments, though from the body weight it would appear that the guinea pigs did suffer a loss of appetite for a day or so after irradiation, the effect on the thymus was negligible. It would appear that there is no abscopal effect on the thymus from abdominal irradiation in the guinea pig

It was calculated that the irradiation dose reaching the shielded thymus was 4% of the total administered amount, i. e. about 16 r which was shown in a previous study (ADAMS, unpublished data) to have no effect on the cellularity of the thymus. Thus, there is no reason to suspect that the shielded thymus in the first and third series of experiments was not functioning normally after irradiation

In the bone marrow of the totally irradiated animal an early depletion of the erythroid and lymphoid populations followed the *pattern which has been described by* HARRIS (9) HULSE (11 12, 13) LAJTHA (15) The recovery pattern in the shielded animals of the first and second experiments also resembles that described by HARRIS (9) when a sub-lethal dose of irradiation was given except that erythroid recovery occurred at a faster rate than that of the lymphoid elements. If the thymus had been influencing this recovery pattern it would be expected that the second series would reveal this influence or the lack of it. Since no appreciable change in the bone marrow occurred when the thymus was missing it seems unlikely that the thymus had contributed anything to this recovery That the recovery of the erythroid cells occurred slightly earlier than those cells of the lymphoid line is not considered of great significance in this study since this also happened in the second series when the thymus had been removed.

From the second experiment it would seem that the thymus did not contribute anything to the recovery seen in the first experiments. The third series was undertaken to exclude the possibility that some factor from the thymus might play a part in the bone

marrow recovery when other elements were not involved. Although the stimulus to any organ capable of causing repopulation of the bone marrow must have been great in the lethally irradiated animal, the shielded thymus apparently contributed nothing and it would appear that there is very little passage of useful cells from the thymus to the bone marrow. FORD and MICKLEM (6) have shown that the reverse can occur, i. e. bone marrow cells can repopulate the thymus of an irradiated recipient. One possibility is that bone marrow contains a more primitive stem cell (18) than that of the thymus and in a particular environment will proceed to lymphocytic differentiation. It is interesting to note that bone marrow will produce erythroid and granuloid but not lymphoid nodules in the spleens of irradiated animals (20). It would appear that the environment in the thymus is better suited to the transformation of bone marrow stem cells to lymphoid lines than that of the spleen.

Cells from the thymus have been shown to produce lymphoid colonies in the spleens of irradiated animals provided that the donated thymus cells had been pre-treated with phytohaemagglutinin (19). Whether or not thymus cells which have been previously stimulated with phytohaemagglutinin could repopulate even the lymphoid elements in irradiated bone marrow is not known.

The beneficial effect of shielding the neck region requires some explanation and the possible sources of the protective agent must be considered. It is likely that some lymph nodes in the neck were shielded but MICKLEM and FORD (21) were unable to show that injected lymphocytes and thymocytes protected against lethal irradiation in mice. Shielding Peyers patch caused a regeneration of thymus in lethally irradiated mice but there was no evidence of granulocytic, megakaryocytic or erythroid regeneration (14). On the other hand SALVIDIO *et al.* (23) by giving large numbers of lymphocytes found substantial survival in lethally irradiated rats. A more likely possible origin of the factors which caused repopulation of the bone marrow in the present experiments is the haemopoetic tissue in the myeloid spaces of the vertebrae (1). The amount of bone marrow protected in this study by the 4 ×2 cm shield would appear small. However using the figure of 8 ×10 cells by FLIEDNER *et al* (7) for protection against irradiation in the rat it would appear that the volume of bone marrow required for protection would be in the region of 40 mm³ since 1 mm of bone marrow contains

approximately 1.8×10^{4} cells (10). No figures are available as to the volume of bone marrow contained in the cervical vertebrae in the guinea pig but it would seem possible that they would contain this amount. An interesting follow up to the present study would be the quantitative analysis of the minimum amount of bone marrow which has to be shielded to allow repopulation to occur.

No evidence was obtained to suggest that a cell free product of the thymus would aid haematopoietic recovery, as has been found in the spleen (5).

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Summary

A lethal dose of irradiation from a cobalt 60 source was administered to guinea pigs while shielding the region of the neck containing the thymus. Shielding protected the animals from irradiation death and bone marrow recovery took place. Thymectomy prior to irradiation did not diminish the protective effect. Shielding the exteriorated thymus did not protect the animals nor was there any recovery of the bone marrow population. It was concluded that thymus cells do not contribute to bone marrow cellularity after irradiation.

Zusammenfassung

Meerschweinchen wurden einer letalen Strahlendosis aus einer Kobalt[60]-Quelle ausgesetzt, während ihre Halsregion mit der Thymusdrüse abgeschirmt wurde. Die Abschirmung schützte die Tiere vor der tödlichen Strahlenwirkung, und das Knochenmark erholte sich. Eine Thymektomie vor der Bestrahlung setzte die Schutzwirkung nicht herab. Eine Abschirmung der nach außen verlagerten Thymusdrüse vermochte die Tiere nicht zu schützen und es trat keine Erholung des Knochenmarkes ein. Aus den Ergebnissen geht hervor, daß Thymuszellen zum Zellbestand des Knochenmarkes nach der Bestrahlung nicht beitragen.

Résumé

Des cobayes furent soumis à une dose létale de rayons venant d'une source de cobalt[60], la région du cou comprenant le thymus étant protégée. Cette protection préserva les animaux de la mort par irradiation, la moelle osseuse se rétablit. Une thymectomie faite avant l'irradiation ne diminua pas l'effet protecteur. De protéger le thymus transplanté ne sauva pas les animaux et la moelle osseuse ne se rétablit pas. Ces résultats indiquent que les cellules du thymus ne contribuent pas à reconstituer la moelle osseuse après l'irradiation de celle-ci.

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Medical Department B, Copenhagen County Hospital, Hellerup (Chief Dr P Rm)

The Nuclear Segmentation of Eosinophils under Normal and Pathological Conditions

S Sparrevohn and H R. Wulff

The significance of the segmentation of granulocyte nuclei is not known but Arneth's hypothesis (2) that the number of nuclear lobes reflects the age of the cell is usually accepted. Variations in the nuclear segmentation of intravascular and emigrated neutrophilic granulocytes have been studied in detail (2 7 10) and demonstration of a neutrophil shift to the left in blood films is of recognised value, since it usually reflects an increased neutrophil turn-over

It is surprising that little information is available about the nuclear segmentation of eosinophilic granulocytes under pathological conditions. We therefore decided to study this detail in eosinophil morphology in a number of patients.

In the literature no systematic study of the nuclear segmentation of eosinophils was found. According to Undritz (9) shift to the right in the eosinophil picture is *encountered as rare hereditary anomaly as well as in pernicious anaemia, Hodgkin's* disease and in allergic conditions. Gross (4) on the other hand mentions that eosinophilia is accompanied by shift to the left rather than by shift to the right and in case of suspected penicillin allergy Tudoranu *et al.* (8) observed many immature eosinophils. I cases diagnosed as eosinophilic leukaemia immature forms have been seen in the peripheral blood, but hypersegmentation of the nuclei has also been described (1 5)

Clinical Material

The material (Table I) consisted of normal persons and patients belonging to one of the following three groups (1) bronchial asthma associated with eosinophilia (range 425—7000 eosinophils/μl); (2) eosinophilia of different aetiology (range 421—1250 eosinophils/μl) and (3) ulcerative colitis.

The patients with ulcerative colitis presented normal or in three cases, elevated eosinophil counts (maximum 1200 eosinophils/μl) I this disease, however an increased eosinophil turn-over was expected, since the inflammatory exudate in the colon in 80% of cases contains numerous eosinophils (6)

Table I
Clinical material.

Diagnosis	No.
Normal subjects	14
Bronchial asthma	15
Ulcerative colitis	10
Chronic granulocytic leukaemia	1
Epidemic hepatitis	1
Penetrating eye injuries	2
Systemic disease with pulmonary infiltrates	1
Schistosomiasis	1
Total	45

Methods

Leucocyte concentrates were prepared by modification of EFRATI and ROZENZAJN method (3) 8 ml of blood, stabilized with oxalate, was centrifuged for 15 min at 1500 rpm immediately after venepuncture. The plasma was removed and the leucocyte layer with the adjoining erythrocytes were drawn into glass tube (diameter 4 mm) with pipette tip, which subsequently was sealed with rubber cap. The tube was centrifuged for 6 min at 1800 rpm and column of leucocytes was formed. The glass was broken above the leucocyte column, and using pipette the leucocytes were transferred to watch glass to be mixed with one or two drops of plasma.

Films were made, which were stained using the MAY-GRÜNWALD-GIEMSA method, and the nuclear lobes of 100 or 200 eosinophils were counted. The criteria used were those previously described (7). For each patient lobe index indicating the average number of nuclear lobes per eosinophil was calculated. All preparations were counted by one author and control counts on coded preparations were performed by the other author Films from 4 normal subjects and 6 patients were also stained for glycogen using the hexamine-silver technique (11)

In addition skin window experiment (7) was performed on one patient with bronchial asthma presenting pronounced eosinophilia (7000 /µl) The lobe index was determined in the 4 5 and 6 hour 'skin window' preparations.

Results

In the normal subjects the lobe index averaged 2 06 (range 1 93 to 2 14) 70—90 / of the eosinophils were bilobed and the rest were either unsegmented or 3-lobed. 4-lobed nuclei were rarely encountered (Fig 1 and 2a)

In the patients with bronchial asthma, ulcerative colitis and eosinophilia from other causes the lobe index was clearly increased, although pronounced variation was seen (Fig 1) Most patients presented many eosinophils with three nuclear lobes, and 4-lobed nuclei were frequently encountered (Fig 2b) Only four patients

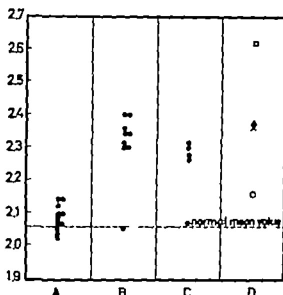


Fig 1 Lobe index of eosinophilic granulocytes. *(A)* Normal subjects. *(B)* Cases of bronchial asthma with eosinophilia. *(C)* Cases of ulcerative colitis. *(D)* Cases with eosinophilia of different aetiology

□ schistosomiasis (lobe index determined in ordinary blood film)
▲ chronic granulocytic leukaemia
△ systemic disease with pulmonary infiltrates
O penetrating eye injuries
epidemic hepatitis

had a lobe index overlapping the normal range No correlation was observed between the increase in the eosinophil count and the degree of the shift to the right

The skin window experiment which was performed on a patient with bronchial asthma revealed numerous emigrating eosinophils in the skin lesion. This patient presented a lobe index of about 2 40 in the blood by repeated determinations, but in the exudate the lobe index was about 2.0 (4 hour preparation 1 96 5 hour preparation 1 90 and 6 hour preparation 2 00)

No difference was observed between the glycogen content of the eosinophils in normal subjects and in 6 patients presenting increased lobe indices.

Discussion

A shift to the right in the eosinophil blood picture was observed both in patients with eosinophilia regardless of the aetiology

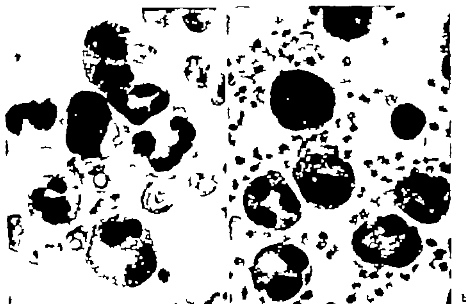


Fig. .. Leucocyte concentrates. *( )* Normal subject. Two eosinophils (at the top and at the bottom) with bilobed nuclei. *(b)* Patient with bronchial asthma. Six eosinophils with 2 to 4 nuclear lobes.

and in patients with ulcerative colitis where previous studies have shown that the eosinophils are mobilised (6). These observations were unexpected since mobilisation of the neutrophil system is accompanied by a shift to the left.

If ARNETH's hypothesis is correct the eosinophil shift to the right reflects an increased average age of the blood eosinophils. That would be the result, if exclusively the younger less segmented forms left the blood vessels by emigration. This explanation is supported by the skin window experiment, where almost all emigrated eosinophils were bilobed, in spite of the pronounced shift to the right in the blood. By contrast neutrophilic granulocytes present hypersegmentation after emigration (7 10).

It is, however, also possible that activation of the eosinophil system is accompanied by an accelerated nuclear segmentation of the eosinophils.

A shift to the left of the eosinophil picture was not observed in any of the patients, but the observation made by TUDORANU *et al* (8) suggests that it may occur in acute allergic reactions.

The determination of the lobe index of eosinophilic granulocytes is time-consuming and can therefore not be used as a routine procedure. The present results suggest however that the procedure may be helpful in elucidating the role played by the eosinophil in disease states

Summary

The nuclear segmentation of eosinophilic granulocytes was studied in leucocyte concentrates from normal subjects and patients with bronchial asthma, eosinophilia of other aetiologies and ulcerative colitis. In the normal subjects the great majority of the eosinophils contained two nuclear lobes, whereas the eosinophils from the patients presented a varying 'shift to the right'. A skin window experiment suggested that the shift to the right was caused by selective emigration of less segmented eosinophils.

Zusammenfassung

Die Kernsegmentierung eosinophiler Leukozyten wurde in Leukozytenkonzentraten von Gesunden und von Patienten mit Bronchialasthma, mit Eosinophilie anderer Aetiologie und mit Colitis ulcerosa untersucht. Bei Gesunden enthielt die grosse Mehrzahl der Eosinophilen zwei Kernsegmente, während bei den Patienten die Eosinophilen eine verschieden starke Rechtsverschiebung aufwiesen. Ein Hautfenster-Versuch ergab, dass die Rechtsverschiebung durch eine selektive Auswanderung weniger segmentierter Eosinophiler bedingt war

Résumé

La segmentation du noyau des granulocytes éosinophiles a été étudiée dans des concentrés de leucocytes venant de personnes saines et de malades atteints d'asthme bronchique, d'éosinophilie d'autres étiologies et de colite ulcéreuse. Chez les sujets sains, la grande majorité des granulocytes éosinophiles contenait un noyau bilobé. Les éosinophiles des malades présentaient une déviation vers la droite. Une expérience à l'aide d'une «fenêtre dans la peau» démontra que la déviation vers la droite est due à une émigration sélective d'éosinophiles dont le noyau est moins segmenté

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Medizinische Abteilung des Kreisspitals Männedorf-Zürich
(Chefarzt Prof. Dr. med. C. Maier)

Hämolytische Anämie bei Sarkoidose der Milz

S. Wyss und C. Maier

In den letzten 20 Jahren sind vereinzelte Fälle von Boeckscher Sarkoidose bekannt geworden, in deren Verlauf eine hämolytische Anämie aufgetreten ist und Fälle von hämolytischer Anämie, bei deren Abklärung – manchmal auch erst post mortal – eine Sarkoidose entdeckt werden konnte. Vermutlich gehören schon die Fälle von Haden (4) und Kracke und Hoffman (6) dazu, während von Crane und Zetlin (2) eine der ersten ausführlichen Beschreibungen eines hämolytischen Ikterus bei generalisierter Sarkoidose mit Splenomegalie stammt. Wegen der Seltenheit hämolytischer Anämien bei Sarkoidose und der Probleme, die durch eine gleichzeitige Splenomegalie aufgeworfen werden, soll eine eigene Beobachtung mitgeteilt und zusammen mit 9 Mitteilungen der Literatur diskutiert werden.

Eigene Beobachtung

D. J., geb. 1928. Weder in der Familie noch in der Vergangenheit des Patienten sind Fälle von Gelbsucht oder Anämie bekannt. 1956 BCG-Impfung. Erstmals 195 anläßlich einer Reihendurchleuchtung Feststellung vergrößerter *Hilusdrüsen*. Derselbe Befund wurde 1958 erhoben. Zunahme der Drüsenvergrößerung und Hinzukommen von Lungeninfiltraten, besonders im rechten Oberfeld gaben 1960 Anlaß zur *Sanatoriumsbehandlung* wegen *Verdacht auf Tuberkulose*. Bei wenig beeinträchtigtem Allgemeinzustand war die Blutsenkung auf 7 mm erhöht, und bei normaler Leukozytenzahl bestand eine Eosinophilie von 9% und Monozytose von 12%. Während der zehnwöchigen Behandlung erhielt der Patient 13 g Rimifon, 831 g PAS und 62 g Streptomycin nebst Prednison. Tuberkelbazillen konnten keine nachgewiesen werden. Über allfällig durchgeführte Tuberkulinreaktionen konnten wir keine Angaben erhalten. Senkung und Blutbild normalisierten sich schon innerhalb eines Monats (Prednisoneffekt), während der Lungenbefund nur teilweise besserte. 1960 ergab eine Thoraxkontrolle Progression des Lungenprozesses. Die auf dieser Röntgenaufnahme sichtbare Milz ist noch klein. Nun wurde eine *Biopsie nach Daniels* gemacht. Sie ergab eine *epitheloidzellige Granulomatose ohne Verkäsung*. Der Patient entzog sich aber weiteren Kontrollen und begab sich in naturärztliche Betreuung. Er fühlte sich gesund und ging seiner Arbeit nach.

2 Jahre später, am 4. Mai 1963, trat ein leichter *Ikterus* in Erscheinung; der Hausarzt dachte an eine Hepatitis und behandelte den Patienten drei Wochen lang mit Bettruhe, Diät, Vitaminen. Bilirubin 8,0 mg%, Transaminasen im Serum nicht erhöht. Deshalb erfolgte zur weiteren Abklärung am 30. Mai 1963 Spitalaufnahme.

Medizinische Abteilung Kreisspital Männedorf 31. Mai bis 27. Juni 1963. Die führenden Befunde bei Spitalaufnahme waren: hochgradige *Anämie*, *Ikterus*, vergrößerte *hiläre Drüsen* mit beidseitigen *Lungenparenchymveränderungen* und einwandfreie *Vergrößerung der Milz*. Die Austestung mit Tuberkulin (bis Mantoux mit PPD 100 E) ergab eine negative Cutanreaktion. Zusammen mit dem früher erhobenen Befund in einer Supraklavikulardrüse interpretierten wir das Krankheitsbild als *Sarkoidose mit Splenomegalie und erworbener hämolytischer Anämie*.

Klinisch fanden sich keine weiteren Stationen mit Sarkoidose. Entsprechend dem hohen Retikulozytengehalt bestand eine Senkungsbeschleunigung von 32 mm. Hämoglobin 48% (hämatologische Daten siehe Tabelle I). Bilirubin 4,7 mg%. Prothrombinzeit nach Quick 83%. Serumeisen 178 γ%, alkalische Phosphatase 2,0 mMol E., SGO-Transaminase 74 E, SGP-Transaminase 32 E, Blutzucker enzymatisch 74 mg%, Cholesterol 105 mg%, Calcium 10,0 mg%, Galaktosebelastung normal. Urin chemisch und mikroskopisch unauffällig.

Serologische Luesreaktionen, Agglutinationen auf Brucellen, Salmonellen negativ. LE-Test negativ. Im Ekg Sinusrhythmus, Vitalkapazität 87%, Atemgrenzwert 54% der Sollwerten.

Nach Operationsvorbereitung mit insgesamt 2000 ml Blutkonserven wurde am 11. Juni 1963 eine 630 g schwere *Milz exstirpiert* (PD Dr. P. Ricklin, Chefarzt, chirurgische Abteilung).

Wie aus der Darstellung in Abb. 1 hervorgeht, normalisierten sich die hämatologischen Verhältnisse nach der Splenektomie sehr schnell. Der leichte Ikterus verschwand. Aufgrund der weiteren Verlaufsbeobachtung während des Zeitraums von 1½ Jahren erweist sich die hämolytische Anämie als definitiv behoben. Nicht nur ergaben sich 9 Monate nach Splenektomie normale Verhältnisse in der osmotischen Resistenz, sondern auch die Bestimmung der *Erythrozytenlebensdauer* mittels Chrommarkierung 1½ Jahre nach dem Eingriff zeigt eine normale biologische Halbwertszeit (Tabelle I). Die pulmonale Sarkoidose verhält sich stationär, die Lungenfunktionsprüfung ergibt normale Werte. Wir verdanken die Nachuntersuchung Herrn Prof. Dr. H. Herzog, Bürgerspital Basel.

Die exstirpierte *Milz ist von Sarkoidose befallen*. Das 630 g schwere Organ zeigt makroskopisch auf Schnitt multiple kleinste, nicht unter sich konfluierende Knötchen. Histologisch handelt es sich um zahlreiche grössere und kleinere produktive Epitheloidzellgranulome mit Langhansschen Riesenzellen, bei Fehlen von Nekrose und nur geringer Tendenz zu Fibrose. Die *Lymphknoten des Milzhilus* weisen denselben Befund auf, während bei der Operation entnommenes *Lebergewebe* sarkoidosefrei ist und lediglich eine auf die durchgemachte Hämolyse zurückzuführende leichte Hämosiderose erkennen lässt. (Pathologisches Institut der Universität Zürich, PD Dr. Schmidmann)

Diskussion

Wir haben in der Literatur insgesamt 9 Fälle von Boeckscher Sarkoidose finden können, bei denen im Verlauf der Krankheit eine hämolytische Anämie in Erscheinung getreten ist; sie sind in Tabelle II (Angaben zur Sarkoidose) und Tabelle III (hämatologische Daten) zusammen mit der eigenen Beobachtung dargestellt.

Tabelle I
Hämatologische Werte.

	vor Splenektomie 31. Mai 1963	16 Tage nach Splenektomie 27. Juni 1963
Hämoglobin g%	7,8 (= 48%)	12,6 (= 79%)
Erythrozyten	1900000	400000
Retikulozyten, ‰	354 + 3 Einpunktige	19 + 39 Einpunktige
Howell-Jolly-Körperchen	keine	+
Mikrosphärozytose	+++	verschwunden
Hämatokrit, %	24	34
Färbeindex	1,26	0,95
DVE μ^3	125	84
Hb E γγ	41	29
HbKE	33	41
Leukozyten	4500	3100
Stabkernige, %	49	26,5
Segmentkernige, %	30	17
Eosinophile	3	13,5
Basophile, %	2	3,5
Monozyten,	8	13
Lymphozyten	8	24,5
Thrombozyten	180000	1100000
Sternalmark, Eisenfärbung	EP massiv gesteigert. Ganz vereinzelte Sideroblasten, keine vermehrten Eisenniederschläge	
Direkter Coombstest Pat. Erythrozyten	negativ	
Kälteagglutinine Pat. Serum	negativ	
Wärmehämolysine Pat. Serum	negativ	
Säure-Kältehämolysine Pat. Serum	negativ	
Donath-Landsteinerhämolysine	negativ	
Osmot. Resistenz der E.	0,86–0,24 NaCl	0,52–0,24 NaCl
Wärmeresistenz der E.	stark pathologisch	normal
Lebensdauer der E. (biolog. Halbwertszeit der Cr^{51} markierten E.)		gemessen 30 Tage (Normwert 25 bis 31 Tage)

Zentrallaboratorium, Blutspendedienst Schweizerisches Rotes Kreuz, Bern (Dr. R. Bütler)
Dr. H. P. Braun, Medizinische Universitätsklinik Basel.

Zur Sarkoidose

Die Diagnose der Sarkoidose der 10 Fälle wurde aufgrund klinischer und in 9 Fällen histologischer Kriterien gestellt, in 7 Fällen (Nr. 2, 3, 4, 6, 7, 9 und 10) im Rahmen der Abklärung der Anämie bzw. durch die Splenektomie, in zwei Fällen (1 und 8) jedoch erst

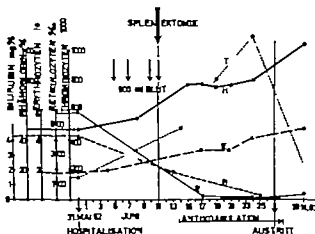


Abb. 1. Darstellung der hämatologischen Daten vor und nach Splenektomie.

post mortem. Über die Beobachtungsdauer der Sarkoidose bis zum meist abrupten Auftreten der Anämie existieren nur für 5 Fälle Angaben (Nr. 2: 6 Wochen, Nr. 3: 2 Jahre, Nr. 6: 2 Jahre, Nr. 9: 25 Jahre, Nr. 10: 6 Jahre). Bei 3 Fällen (Nr. 3, 6 und 7) befand sich die Sarkoidose im Zeitpunkt der Anämie in einer evolutiven Phase, während der entsprechende Befund im Falle Nr. 8 eher stationär war. In unserem Fall (Nr. 10) war die Milz auf einer Thoraxaufnahme zwei Jahre vor der hämolytischen Komplikation noch nicht vergrößert.

Die Veränderungen der Milz sind von besonderem Interesse. In 9 Fällen war die Milz vergrößert (450–1100 g), jedoch nur in 5 von 8 Fällen, bei denen das Organ histologisch untersucht wurde, konnte der eindeutige Befund einer Milzsarkoidose festgestellt werden, wobei die Intensität des Befalls recht unterschiedlich war. Sarkoidose der Lungen, bzw. intrathorakaler Drüsen, war gleichzeitig bei drei von fünf Fällen mit Milzsarkoidose nachweisbar, während in drei Fällen von Lungen- bzw. intrathorakalem Befall der Drüsen die vergrößerte Milz histologisch frei von Granulomen war.

Ist die Sarkoidose an sich keine häufige Krankheit (in England rechnet man aufgrund radiologischer Reihenuntersuchungen auf 100000 Individuen mit 20–30 Fällen = 0,1–0,3‰) (10), so ist die Sarkoidose der Milz noch viel seltener: nach Wyss (17) fanden sich unter etwa 1000 Splenektomien, die seit 1930 in Ohio wegen hypersplenischen Syndromen durchgeführt worden waren, nur 6

Tabelle II

Klinische und pathologisch-anatomische Daten zur *Sarkoidose* bei 10 Fällen mit hämolytischer Anämie. (9 Fälle der Literatur 1 eigener Fall.)

Organbeteiligung der Sarkoidose nach klin. (k) röntgenolog. (r) pathol.-anat. Daten. (*A* = Autopsie *B* = Biopsie *Sp* = Splenektomie), > = vergrößertes Organ. ? = Angabe fehlt.

N	Autoren Jahr	Alter Geschl. (m/f) Rasse (w/n)	Lymphknoten: zervikal	Lymphknoten: supraklavik.	Lymphknoten: axillär	Lymphknoten: ileal	Lymphknoten: retroperit.	Lymphknoten: paratrach.	Lymphknoten: tracheobronch.	Lymphknoten: mediastinal	Lungenhilus	Lunge	Tränendrüse	Auge	Speicheldrüse	Handknochen	Leber	Milz	Nebenmilz (m)	Sternalmark	Milz Gewicht, Histologie
1	Crane und Zetlin, 1945	46/f/w				+ A			+ A			?						— Sp		+ A	485 g Starke Kongestion der Sinusoide, kleine Follikel, weit separiert. Keine Sarkoidose
2	St[illegible] et al., 1947	8/f/n			+ B		> k					—		+ k		—	> k	> k			Milz überragt Rippenbogen um 4 cm. Keine Intervention, keine Histologie
3	McCort et al. 1947	58/m/w						+		+ B		+				+		— Sp		— B	Keine Gewichtsangabe. Milz vergrößert, frei von Sarkoidose. Histologie vereinbar mit Hämolyse
4	D[illegible] und Howe 1950	45/f/n				+ Sp						— r					+ B	+ Sp			500 g. Bild ähnlich kongestiver Splenomegalie b. Pfortaderobstrukt. Pulpa: Stränge von Eosinophilen. Zahlreiche Langhanssche Riesenzellen. Nirgens Nekrose
5	Schumacher 1952	16/m/w										+						?			Keine Angaben über Milz
6	Davis et al., 1954	34/m/n	+ B									+			+ B			+ Sp	+ Sp		450 g, dazu 3 Nebenmilzen mit gleicher Histologie: Diff. Durchsetzung der Milz mit Epithelzellgranulomen, zahlreiche Langhanssche Riesenzellen. Keine Nekrose
7	Lebacq et al., 1956	23/f/w	— k	— k	— k							—	+ B					+ Sp			1100 g Beträchtl. Proliferation enormer Knoten gebildet aus Epitheloidzellen, wenig Lymphozyten, eingestreut Riesenzellen. Fehlen der geringsten Verkäsung
8	Johansson, 1958	59/f/w									+ A	+ A					> A	— A			900 g, in Leber und Milz einige Epitheloidzellherde u. extramed. Blutbildungsherde; keine Sarkoidose
9	W[illegible] 1959	32/f/w									+ B	—					+	+ Sp			530 g Histolog. Boecksche Knötchen in Milz, isolierte granulomat. Knötchen auch in Leber
10	eigener Fall	33/m/w		+ B		+ Sp					+	+					— Sp	— Sp		— B	630 g. Pralle Blutfüllung der Pulpa. Zahlreiche Epitheloidzellgranulome mit Langhansschen Riesenzellen. Nirgens Nekrose, nur geringe

Fälle von Sarkoidose mit Milzbeteiligung. 5 davon hatten eine thrombozytopenische Purpura, ein weiterer eine kongenitale hämolytische Anämie. Ferner befanden sich dort unter den zwischen 1937 und 1939 durchgeführten Autopsien 17 Fälle von Sarkoidose, davon 11 mit Milzbefall, jedoch bestand bei keinem eine hämolytische Anämie.

Zur Anämie

Steht einmal ihr hämolytischer Charakter fest, so bestehen folgende Möglichkeiten:

1. Akzidentelles Zusammentreffen von Sarkoidose und Hämolyse, wie das im Fall Nr. 2 von STATS und ROSENTHAL (15) diskutiert worden ist.:

Bei einem hereditär nicht belasteten Negerkind trat im Alter von 6 Monaten eine schwere hämolytische Anämie auf; es bestand keine Sphärozytose. Nach einer Remission von 7 Jahren kam es, kurz nach Auftreten einer doppelseitigen Iridozyklitis, zu einem schweren Rückfall, diesmal mit ausgeprägter Sphärozytose. Festgestellt wurde nun auch eine generalisierte Sarkoidose, die sicher in der ersten hämolytischen Phase noch nicht vorhanden gewesen ist.

2. Immunhämolytische Anämie bei Sarkoidose. Sie definiert sich heute durch den Nachweis von kompletten oder inkompletten Antikörpern im Serum.

a) Die 7 nach 1950 beobachteten Fälle wurden im Hinblick auf Autohämantikörper untersucht; viermal (Nr. 5, 7, 8 und 9) wurden solche gefunden.

b) Es gibt Fälle, die zusätzliche Panagglutinine, wie Anti T aufweisen (Nr. 8). Die Pathogenese dieser Fälle ist genau so wenig bekannt oder erklärlich wie bei anderen immunhämolytischen Anämien. Immerhin darf aus dem Zusammentreffen der beiden Krankheiten auch auf eine kausalgenetische Verknüpfung geschlossen werden. Bei diesen Fällen brachte die Splenektomie (Tabelle II) nur eine quantitative Besserung der Hämolyse, jedoch keine Heilung, wie dies auch bei anderen immunhämolytisch erworbenen Anämien durchaus bekannt ist.

3. Hämolytische Anämie ohne Nachweis von Antikörpern. Als Illustration dient unser eigener Fall (Nr. 10). Es wurde nach Splenektomie vollständige Heilung erreicht. Hier muss angenommen werden, daß wahrscheinlich nur die *Hypersplenie*, jedoch nicht die Sarkoidose für die Hämolyse verantwortlich ist, ähnlich wie Hämolyse bei

Tabelle III

Hämatologische und einige chemische Daten bei 10 Fällen von Sarkoidose mit hämolytischer Anämie (Werte vor der Therapie im Zeitpunkt des tiefsten Hämoglobins) N der Fälle siehe Tabelle II

Nr	Hb Ez Mill. Reti ‰	Lc Thrombo	Ez qualitat. Sphärozyt. Mikroz.	E-Form im Mark	Untersuchungen zur Hämolyse (OR = osmot. Resistenz)	Erfolg der Splenektomie	Blutchemie E = Gesamteiw., Alb/Glob.
1	41% 1,81 440	15600 150000	+ Normoblast +	Erythro- und normoblastische Hyperplasie	OR 0,32–0,38	Normalisierung, nach 2 Mt. Rezidiv und Tod in erythrobl. Schub	E 5 4→7,6 A 3 6→3 6 G 1,8→4 0
2	37% 3,06 bis 150	6600 270000	+ +	2,4% Erythroblast. 60,8% Normoblast.	OR 0,56–0,28 –0,20	keine Splenektomie	normal
3	55 2,6				Leicht erhöhte Fragilität der Ez	Kein wesentlicher Effekt	E 7,3 g Hyperglobulinämie
4	3,2 g 1,80 220	1900 274000		Hyperplasie der EP mit tel Megalobl.	OR in normalen Grenzen. Keine Kälte-, Wärme- Autoagglutinine. Test auf Sichelzellen neg	8 Monate lang besser dann Rückfall d. Anämie	A und G norm.
5	? 1,50 vermehrt				Direkter Antiglobulintest + + indirekter neg. Kälteagglut.-titer mit 8 normal	keine Splenektomie	
6	6,1 g 1 17 120	6800 204000	keine Sichelzell.	ausgeprägte normoblast. Hyperplasie	OR 0,48–0,18. Coombs dir : neg. Kälteagglut. : neg. Plasmacisen Turnover 2,14 mg/kg/Tag (5 × erhöht)	Ohne Einfluß auf Hämolyse LG zu Corticon	E 6,3 g G γ 1,29 g%

7	32% 1,62 236	10700	Erythrobl.		EP aktiviert	OR 0,42–0,32. Coombsdir : ++ Autoagglut. 4 0; 37 ++ (bis 1 64 (autoglobules trypsinisés en milieu plasma albumine")	Zuerst Hypersplenisyndrom Häm. Anämie 6 Mt. nach Splenekt.	E 6,2 g A 40,2% G 29,0%
8	24% 0,9 407	30000 409000				OR 0,90–0,44. Coombs dir +/ Indir schwach + Tests Hams, Crosby Donath-Landsteiner Kälteaggl., Wärmeaggl. neg. Gewaschene Ec aggl. in versch. Sera z. B. AB-Serum, Anti-T Sera	Keine Splenekt. (Autopsie)	A 7,2 g G γ 29%
9	6,0 g 1 41 298	12100 414000			EP ausgeprägt hyperplastisch) (normoblastisch)	Coombs dir +/indir neg Fäkales Urobilinogen 402 mg/ 24 Std. (vermehrt) Bilirubin 2,9 mg%, davon 2,0 indir	Schnelle Remission (Beobacht. 5 Wo.)	E 6,5 g A 3,9 G 2,6
10	7,8 g 1,9 554	4500 180000	+	+	EP massiv gesteigert, normoblastär viel Makroblasten	OR 0,86–0,24 Coombs dir neg Wärmeresistenz 80,4 mg% (path.). Kälteaggl., Wärmehämolysine, Säure-Kältehämolys., Donath-Landst.-hämolysine nicht nachweisbar	Vollständige, schnelle Remission, OR nach 1 Jahr normal	

splenomegaler Zirrhose. Genau wie Splenomegalien anderer Genese kann offenbar auch eine Milzsarkoidose ohne Hämolyse bestehen.

Es kommt auch vor, daß die hämolytische Anämie zunächst als idiopathisch erworbene Form imponiert, weil die begleitende Sarkoidose erst in einem späteren Zeitpunkt oder erst autoptisch entdeckt wird.

Im Fall Nr. 3 wurde eine latente kongenitale hämolytische Anämie mit Splenomegalie angenommen, da die exstirpierte Milz histologisch unauffällig war; erst ein Jahr später wurde eine ausgedehnte Sarkoidose verifiziert. In andern Fällen führte der histologische Befund der zur Beeinflussung der Anämie entfernten Milz zur Diagnose (Tab. III), während im Fall Nr. 8 durch die Autopsie überraschenderweise eine Sarkoidose entdeckt wurde.

Wie bei andern Prozessen (z. B. Lymphogranulomatose) läßt sich bei der Sarkoidose in einzelnen Fällen (Nr. 5, 7, 10) eine Abhängigkeit des hämolytischen Geschehens von Ausdehnung und Aktivität der Grundkrankheit erkennen. Die hämolytische Anämie setzt offenbar in der Mehrzahl der Fälle ziemlich unvermittelt ein, oft zunächst unter dem klinischen Bild eines leichten Ikterus. Die niedrigsten Hämoglobinwerte liegen um 5g % und sinken wegen der meist kräftig reaktiv gesteigerten Erythropoese selten weiter ab. Von 5 Fällen bestehen Angaben zum roten Blutbild, jedesmal mit Hinweis auf eine Mikrosphärozytose. Die osmotische Resistenz wurde bei 8 Fällen geprüft, ergab aber nur bei zwei eine eindeutig erhöhte Fragilität der Erythrozyten.

Der Erfolg einer Splenektomie ist auf Grund dieser Beobachtungsreihe schwer vorauszusagen. Sechsmal wurde der Eingriff zur Beeinflussung der Anämie vorgenommen: zweimal ohne jeden Erfolg (Fälle 3 und 6), wovon bei letzterem mit negativen immunologischen Befunden die Splenektomie erfolglos war im Gegensatz zur günstigen Wirkung von Cortison auf die Hämolyse. Zweimal wurde nur eine vorübergehende Besserung der Hämolyse erzielt (Fälle 1 und 4), einmal mit völliger Remission, aber einer zu kurzen Nachbeobachtung von nur 5 Wochen (Fall 9). In Fall Nr. 5 mit günstigem Verlauf der Grundkrankheit wurde eine spontane Besserung der immunhämolytischen Anämie beobachtet.

Im eher außergewöhnlichen Fall Nr. 7, bei welchem zunächst die von Sarkoidose befallene Milz wegen eines primären Hypersplenie-Syndroms (Anämie, Leukopenie, Neutropenie) entfernt werden mußte, bildete sich erst sechs Monate später eine auto-

immunhämolytische Anämie aus, wobei Hydrocortison die Hämolyse zu verhindern vermochte.

Aus dem bisher Gesagten geht hervor, daß bei der hämolytischen Anämie bei Sarkoidose die vergrößerte Milz keine einheitlich definierte oder ausschließliche Rolle spielt: einzig unser Fall 10, evtl. auch 9, wurde durch Splenektomie geheilt. Ferner zeigten die vier Fälle mit übrigens quantitativ recht unterschiedlichem Sarkoidosebefall der Milz im Hinblick auf den Effekt der Splenektomie divergierendes Verhalten. Gerade in Fall 6, wo der Milzbefall recht ausgeprägt war und mit der Milz reichlich sarkoidöses Gewebe entfernt werden konnte, blieb die hämolytische Anämie unbeeinflußt. Aber schließlich hat das gesamte retikuloendotheliale System die Milz als bedeutsamer Teil desselben, immunhämatologische Potenzen. Die Frage, auf welche Weise diese durch die Sarkoidose, insbesondere gegen die Erythrozyten, stimuliert werden können, ist nur eine unter vielen. Nicht ohne weiteres können analoge Situationen etwa bei Tuberkulose oder Lymphogranulomatose, mit derjenigen der Sarkoidose verglichen werden, welche besondere *immunologische Probleme bietet. Hingewiesen sei nur auf das* Phänomen der temporären Anergie (Tuberkulinallergie usw.) oder auf die von Lebacq und Verhaegen (7) nachgewiesene Möglichkeit der passiven Übertragung der Kveimschen Reaktion auf gesunde Individuen mit Leukozyten von Sarkoidoseträgern. Dazu wissen wir auch noch nicht, was die Sarkoidose ist, da sie wahrscheinlich auch keine Sonderform der Tuberkulose ist (14, 16 u. a.)

Zusammenfassung

Bericht über einen 35jährigen Mann, welcher während 5 Jahren wegen Sarkoidose der Lungen kontrolliert wurde und dann mit Gelbsucht erkrankte. Es fanden sich alle Zeichen einer hämolytischen Anämie bei negativem Coombs-Test bei Splenomegalie. Die Milz wurde entfernt und wog 630 g. Histologisch fand sich eine Sarkoidose der Milz. Nach der Operation verschwand die Hämolyse vollständig, als seltenes Resultat im Vergleich mit den bisher in der Literatur beschriebenen neun Fällen von Hämolyse bei Sarkoidose.

Summary

The case is reported of a 35-year-old man who was observed for five years because of sarcoidosis of the lung and then presented with jaundice. All the signs pointed to haemolytic anaemia, with negative Coombs test and splenomegaly. The spleen was removed and found to weigh 630 g. Histological examination showed sarcoidosis of the spleen. Post-operatively the haemolysis disappeared completely. The nine cases of haemolysis occurring in sarcoidosis so far reported in the literature are discussed.

Résumé

Rapport du cas d'un homme de 35 ans qui pendant 5 ans a été en observation pour une sarcoïdose des poumons et qui alors fut atteint d'une jaunisse. Tous les signes d'une anémie hémolytique furent constatés, le test de Coombs restant négatif et la splénomégalie faisant défaut. La rate fut extirpée et pesait 630 g. L'examen histologique révéla une sarcoïdose de la rate. L'hémolyse disparut complètement après l'opération. Les neuf cas d'hémolyse accompagnant une sarcoïdose qui ont été décrits jusqu'à maintenant dans la littérature sont discutés.

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A Case of Idiopathic Aplastic Anaemia Associated with Trisomy 21 and Partial Endoreduplication

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The association of leukaemia with mongolism or trisomy 21 has been observed by several investigators (1 10) The incidence of leukaemia is significantly high in patients with mongolism (1 10) The development of aplastic anaemia in trisomic states, however has not yet been reported.

Recently we have observed a severe case of idiopathic a plastic anaemia, the cytogenetic study of which has revealed trisomy 21 aneuploidy and a partial endoreduplication.

Case Report

F U 17 year old boy was admitted to the Çapa Internal Clinic because of epistaxis, pallor blurred vision and tiredness. Tw months prior to his admission he had had epistaxis ttacks for which he was hospitalized for 8 days in his native city One week ago, severe epistaxis has occurred together with petechiae on his lower extremities and abdomen. There was no history of previous illness except pleurisy He had never used any drugs except vitamins prescribed for his present illness. H has had no contacts with noxious agents.

P E. revealed very pale, well nourished boy with no hair on chest and axillar regions. He was 163 cm high and weighed 77.3 kg. There were petechiae on his lower extremities and lef arm. There was no gynecomastia, lymphoadenopathy and hepatosplenomegaly His testes ere of normal size and elasticity but his penis was smaller than normal Scarce pubic hair was present. His vertex-pubis measured 89 cm, his span 163 cm and his pubis-heel 6 cm. B. P was 114 to 70 mm Hg and his temperature ranged between 36.8 C and 37.8 C. Ophthalmologic examination revealed multiple fresh, preretinal haemorrhagic foci on both sides. There were no cataracts His palmprint was normal.

Laboratory data. RBC 1400000 mm³ Haemoglobin 3.2 g 100 ml Platelets 54720 per mm³ reticulocytes 1.8 hematocrit 11.5 MCV 100 μg. MCHb 29 pg. WBC 2200 mm³ with 6 band forms, 50° neutrophils, 2° monocytes and 42 lymphocytes. The blood film wa made up of normochromic and macrocytic red blood cells with mild anisocytosis and marked poikilocytosis The BSR was 78 mm in one hour (Westergren). The tourniquet test was positiv Bleeding time (Duke wa 7' min and

coagulation time (Lee White) 5 min. The clot did not retract. A sternal marrow aspirate was hypocellular with 7% neutrophil myelocytes, 4% eosinophil myelocytes, 8% neutrophilic band forms, 2% segmented neutrophils, 2% plasmocytes and 50% lymphocytes. The red cell precursors comprised 13% polychromatic normoblasts, 13% orthochromatic normoblasts and 1% basophilic erythroblasts (total 27%). There were no megakaryocytes. The urine urobilinogen was 0.4 Ehrlich units/2 h. Direct Coombs test negative. Blood urea was 23 mg%. Gastric juice analysis revealed hypoacidity. Fasting blood sugar 112 mg%. Serum electrolytes (mg%): chloride 356, potassium 18, and sodium chloride 587. Serum iron 212 gamma%. Serum uric acid 5 mg%, alkaline phosphatase 3.3 Bodansky units, serum phosphorus 4.1 mg%. Urinary 17-keto steroids were 9.45 mg/24 h. Serum paper electrophoresis revealed a mild decrease in albumin and a mild increase in $alpha_2$-globulin fractions with a marked increase in the gamma-globulin fraction. Wassermann and Kahn reactions were negative. The BMR was minus 14. The radioactive iodine uptake revealed no abnormality. The prothrombin time was 15 s. Spermatogram was normal. Haemoglobin analyses revealed a normal HbA_2 and 9% foetal haemoglobin. X-ray examinations of the skeletal system, chest and sella turcica were normal. His I.Q. (Cattell) was 81. Sex chromatin studies (skin biopsy) gave negative results.

Cytogenetic study was performed on a 72-hour culture (199 TC-Difco) of peripheral blood with phytohaemagglutinine added, where the slightly modified technique of Moorhead *et al.* (8) was used. One hundred mitotic figures were counted. All the analyzed cells showed a model number of 47. There was no mosaicism. In the karyogram, a trisomy of chromosome 21 was observed (Denver classification) (6) (Fig. 1 and 2). Sex chromosome constitutions (XY) were normal. In addition to this aneuploidy some of the mitotic figures revealed a partial endoreduplication (Fig. 3).

Course. The patient has been under treatment with corticosteroids, methyltestosterone, whole blood transfusions and phytohaemagglutinine for 18 months. He is now in a good condition and there are no signs of bleeding diathesis although those of hypercorticism such as a moon-face, buffalo-hump, striae etc. are present. Although the patient has received no transfusions in the last two months, the haematologic data has improved moderately. The haematologic data are as follows: WBC 3800 mm³, RBC 2810000 mm³, platelets 98000 mm³, haemoglobin 8.2 gm/100 ml, hematocrit 27.5, MCV 96.4 cu.microns.

Comments

As can be seen from the case report, the clinical features and the haematological findings of the propositus are consistent with the diagnosis of aplastic anaemia. These are: bleeding diathesis, pancytopoenia, hypoplastic bone marrow, slightly increased foetal haemoglobin etc. Neither a toxic agent nor congenital anomalies, except a mild hypogonadism have been detected. Findings such as the absence of hair on the chest and axillar regions, scarce pubic hair and the low level of urinary 17 keto steroids brings forth the probability that the diagnosis of congenital aplastic anaemia is not impossible.

The interesting point in this case was the results of cytogenetic study which revealed the presence of trisomy, aneuploidy and partial endoreduplication.

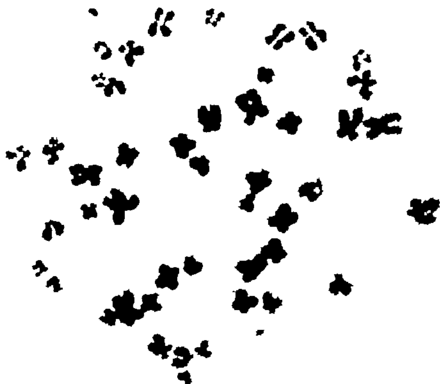


Fig 1 Metaphase plate from the peripheral leukocyte of patient with idiopathic aplastic anaemia showing trisomy-21

Trisomic states are found to be characterized by 47 chromosomes, the extra one being one of the autosomes or non-sex chromosomes. The classic example of this kind of anomaly is mongolism (Down's syndrome) the trisomy 21 (the smallest non-sex chromosome) In our case, the trisomy in the chromosome pattern proves that this case belongs to the group of patients with mongolism only by chromosomal appearance. The patient almost totally lacked the main features of mongolism. The hypogonadic state of the patient can well be explained on the above basis.

The anomaly of endoreduplication of chromosomes, however may not be regarded as a symptom of mongolism but Biesele *et al* were the first to demonstrate endoreduplication of chromosomes in a case of translocation of 15 21 familial mongolism 4) Endoreduplication is encountered primarily in persons with chro-



Fig. — Karyotype from the peripheral leukocyte of a patient with idiopathic aplastic anaemia showing trisomy 21

mosome abnormalities and in their phenotypically normal siblings and parents (11). Valdmanis et al. (11) had indicated that endoreduplication was present in the thirty year old mother and a normal brother of a trisomy 21 mongol boy. Notably, it was not found in normal parents and children. Although endoreduplication was described in association with blood disorders (2, 5, 9), it is well known that endoreduplication is a process of polyploidization occurring during interphase which is characterized by diplochromosomes (7). The amount of endoreduplications in the cell is directly related to the immaturity of the foetus (3).

As endoreduplication has not been observed in cases with aplastic anaemia this anomaly may well be related to the present illness of the patient. This relationship could, therefore, suggest that the aplastic anaemia is perhaps caused by a failure in the reproduction and division of the stem cells of the blood. This statement, however, needs further investigation for sake of proof.



Fig. 3. Metaphase plate from the peripheral leukocyte of patient with idiopathic aplastic anaemia showing polyploidy (endoreduplication)

Addendum. Since submission of the manuscript, we reexamined the propositus who was in very good condition. RBC/4 150 000 mm^3 Hb. 11.2 g/100 ml, platelets 79 000/mm WBC 3600/mm^3 and hematocrit 41 The treatment was stopped 3 months ago.

Summary

A case of idiopathic aplastic anaemia occurring in association with trisomy-21 and endoreduplication is reported. Although the above defined cytogenetic state can be encountered in patients with leukaemia, no such anomaly has yet been observed in aplastic anaemia.

Zusammenfassung

Es wird über einen Fall von idiopathischer aplastischer Anämie berichtet, der eine Trisomie 21 und eine Endoreduplikation aufwies. Diese zytogenetische Anomalie die bei Patienten mit Leukämie vorkommt, wurde bisher bei aplastischer Anämie noch nicht beobachtet.

Résumé

Rapport d'un cas d'anémie aplastique idiopathique comportant une trisomie 21 et une endoréduplication. Cette anomalie cytogénétique qui se trouve chez les malades atteints de leucémie n'a jusqu'à maintenant pas été observée dans l'anémie aplastique.

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Kasuistischer Beitrag zum Krankheitsbild der akuten Eosinophilen-Leukämie

Ch. Hauswaldt, S. Raju, L. Bianchi und W. Hunstein

Die Eosinophilen-Leukämie (EL) ist eine sehr seltene Krankheit. Ihre Existenz als Sonderform der vom granulopoetischen System ausgehenden, maligne proliferierenden Erkrankungen wird aber heute – mit wenigen Ausnahmen (6, 17) – nicht mehr bezweifelt (4, 12). Auch die der akuten Leukose entsprechende Verlaufsform ist nur vereinzelt beschrieben worden [11 der von Dittrich (5) bis 1952 zusammengestellten Fälle, ferner die von Ackermann (1), Gerhard *et al.* (8), Gross *et al.* (13) und Sacrez *et al.* (19)]. Wegen der Seltenheit der akuten EL soll im folgenden ein Bericht über eine eigene Beobachtung gegeben werden, zumal die Existenz dieser klinisch und allgemein pathologisch interessanten Sonderform der akuten Leukämie wenig bekannt ist und wohl auch zu wenig beachtet wird.

S. K., ♂, 75 Jahre alt

Familienanamnese und eigene Vorgeschichte ohne Besonderheiten.

Jetzige Erkrankung: Seit August 1962 allgemeine Schwäche. Im Oktober 1962 vorübergehend Schmerzen und Schwellung des rechten Beines. Im November erneute Schwellung des rechten Beines und rechten Armes, Fieber. Anfang Dezember schmerzhafte Zahnfleischulzerationen. Mitte Dezember hohes Fieber, Nasenblutungen, Schwellung der axillären Lymphknoten. Deswegen am 5. 1. 1963 stationäre Aufnahme.

Aufnahme-Befund: Stark reduzierter Allgemeinzustand, Ruhedyspnoe. Blässe der Haut und sichtbaren Schleimhäute. Am Rücken zahlreiche Hautulzerationen, an den Extremitäten petechiale Blutungen und erythematöse Flecken. An beiden Halsseiten, in der rechten Achselhöhle und in beiden Leisten bis bohnengroße derbe, unverschiebliche Lymphknoten. 1,5 cm große Schleimhautulzeration am Unterkieferzahnfleisch. Vergrößerte, chronisch entzündete Tonsillen. Leber 3 QF, Milz 4 QF unterhalb des Rippenbogens tastbar, derb. An den übrigen Organen kein pathologischer Befund.

Blutbild: Hb 6,0 g%, Ery 1,7 Mill., Hb_E 35 γγ, Retikulozyten 7 ‰, Thrombozyten 32.000, Leukozyten 49.000 (77% Paramyelobl., 3% Promyeloz., 1% Myeloz., 1% Metamyeloz., 1% Stabk., 2% Segmentk., 9% Basoph., 5% Lymphoz.) BSG 127/165



Abb. 1. Sternalmark bei Eosinophilen-Leukose (Eosinophilen-Färbung nach UNDRITZ). Vergr. 1:1320

nem n. W. Coombs-Test negativ. Kälte-Agglutinin-Titer normal. Unauffällige osmotische und mechanische Resistenz der Erythrocyten. Fe 154 γ%, Cu 178 γ%.

Sternalmark (Abb. 1). Hyperplastisches Mark mit Vermehrung der granulopoetischen Vorstufen. Innerhalb der Granulopoese viele Paramyeloblasten, daneben nur spärlich neutrophile Myelocyten und Metamyelocyten. Vermehrung unreifer eosinophiler Zellen mit unregelmäßiger dicker eosinophiler und basophiler Granulation (Färbung nach UNDRITZ). Verminderung der erythropoetischen Vorstufen und der Megakaryocyten.

Lymphknoten-Punktat. Dichte Infiltration mit unreifen Eosinophilen.

Diagnose. Das Blutbild, besonders die hohe Leukocytenzahl mit Auftreten von Paramyeloblasten und die zahlreichen unreifen Eosinophilen im Sternalmark sprechen für eine akute Eosinophilen-Leukämie. Gegen eine leukämoide Reaktion bei einer akuten Paramyeloblasten-Leukose war das Auftreten von zahlreichen unreifen Eosinophilen im Sternal- und Lymphknoten-Punktat zu verwerten.

Verlauf. Es kam zu einer rapiden Verschlechterung des Zustandes. Die Behandlung mit Corticosteroiden (Decortin bis 250 mg/die, Ortostanon), Purinethol 200 mg/die, dann Endoxan 200 mg/die), Bluttransfusionen und Antibiotika konnte den fulminanten Verlauf nicht beeinflussen. Am 15. Tage nach der Klinikaufnahme kam der Patient an einer Bronchopneumonie ad exitum.

Autopsie

Milz (Abb. 2). 655 g. Glatte Oberfläche. Grau-rötliche Schnittfläche mit verwaschener Follikel- und Trabekelzeichnung. Histologisch in den Pulpasträngen dichte Infiltration von myeloischen Zellen, vorwiegend mit Eosinophilen.

Leber (Abb. 3). 2330 g. Glatte Oberfläche. Histologisch erhaltener Läppchenaufbau. Dichte Infiltration der periportalen Felder und fleckenförmige Infiltration der Sinus durch unreife, vorwiegend eosinophil granulierte myeloische Zellen. Die geschwollenen Sinusendothelien enthalten feinkörnige PAS-positive Massen.

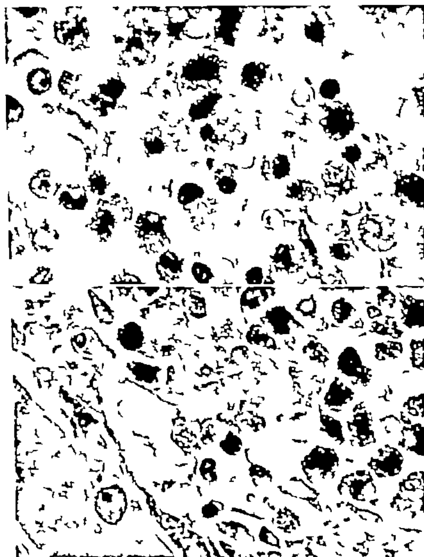


Abb. 2. Eosinophilen-Infiltrat in der Milz. Giemsa. Vergr. 1:1000

Abb. 3. Eosinophilen-Infiltrat in der Leber. H.E. Vergr. 1:1000

Lymphknoten: Am Hilus, mesenterial, paraaortal und inguinal vergrößert. Weich, grau-rote Schnittfläche. Histologisch diffuse Wucherungen von teils rundkernigen, teils gelappt-kernigen Eosinophilen, nur vereinzelt Lymphocytenhaufen.

Nieren: Zusammen 330 g. Oberfläche und Schnittfläche weißgrau verfärbt. Histologisch bei erhaltenem Aufbau in Mark und Rinde Infiltrate von rund- und gelapptkernigen Eosinophilen.

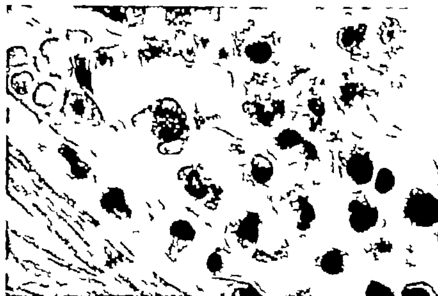


Abb. 4 Eosinophilen-Infiltrat im Herzmuskel (H. E. Vergr. 1 : 1000)

Herz (Abb. 4) 355 g. Rechter Ventrikel und Vorhof ausgeweitet, linker Ventrikel und Vorhof kontrahiert. Einige punktförmige Blutungen am Epicard sowie in Endocard des rechten Ventrikels. Histologisch im Epicard ausgedehnte Infiltrationen von vorwiegend unreifen Eosinophilen, fleckförmige Infiltration auch im Myocard der Außenzone.

Lunge Rechts fibrinöse Pleurabeläge. Zahlreiche, diffus verstreute weißliche Herde mit trübem Abstrichsaft. In den Bronchien grau-gelblicher Schleim. Histologisch fleckförmige Nekrosen mit massenhaft Bakterienrasen, nur spärlich polynukleäre Leukozyten. Locker eingestreut myeloische, meist eosinophil granulierte Zellen.

Knochenmark. In Epi- und Diaphyse des Femurs grau-rotes Mark. Wirbelmark makroskopisch unauffällig. Histologisch dichte Infiltrate von unreifen und reifen Eosinophilen. Nur ganz spärlich sind unreifzellige, nicht granulierte Markelemente eingestreut.

Pathologisch-anatomische Diagnose. Eosinophilen-Leukämie mit Infiltration von Milz, Leber, Lymphknoten, Nieren und Herz. Bronchopneumonie von agranulozytärem Charakter.

Eine starke Vermehrung der Eosinophilen im Knochenmark oder im peripheren Blut ist allein für die Diagnose der EL ebensowenig beweisend wie die Infiltration nichtblutbildender Organe. Hohe Eosinophilen-Werte, ein »*hypereosinophiles Syndrom*« [12] beobachtet man vor allem als Ausdruck allergischer Reaktionen und auf paranitärer Grundlage – »reaktive eosinophile Leukozytosen« früher »eosinophiles Leukämoid« (10, 20, 21, ausführliche Literatur 2, 12) – auch bei Kollagenkrankheiten (6, 17, 21). Eine Infiltration der Milz, Leber und Lunge, auch des Magen-Darm-Traktes und sogar des Herzmuskels wird hierbei ebenso wie bei der

familiären Eosinophilie gefunden (11 16) Die vielfach beschriebenen Granulations-Anomalien und Zytoplasma Vakuolen der eosinophilen Leukozyten im peripheren Blut und im Knochenmark sind ebenfalls nicht beweisend für eine EL (12) So wurden bestimmte Kriterien erarbeitet, die für die Diagnose einer EL erfüllt sein müssen (3 4 12, 13)

1 Das Leiden entsteht autochthon und führt von sich aus zum Tode – meist, aber nicht immer in einem terminalen Blastenschub

2. Die Eosinophilie beträgt mindestens 50 %.

3 Infiltrate eosinophiler Zellen finden sich generalisiert im Körper

In unserem Fall konnten wir eine Ausschwemmung der Eosinophilen ins periphere Blut nicht beobachten, hier fanden wir überwiegend Paramyeloblasten. Im Knochenmark (Abb. 1) wie auch in den Organ-Infiltraten (Abb 2–4) überwogen aber die *unreifen* Eosinophilen. So ist ein hypereosinophiles Syndrom bei primärer Paramyeloblasten-Leukose unwahrscheinlich. Möglicherweise war es in Analogie zu den Beobachtungen von EVANS *et al* (7) und LAKOS *et al* (15) nach der initialen Eosinophilen-Vermehrung im peripheren Blut zum Blasten-Schub gekommen, von dem wir nur die terminale Phase beobachteten.

Die EL wird als eine von differenzierten granulopoetischen Zellen ausgehende Leukose der Basophilen Leukämie und der leucémie myélogène à polynucléaires neutrophiles zur Seite gestellt (5) Die Annahme von GOH *et al* (9) die chronische Verlaufsform wegen des Fehlens des Philadelphia-Chromosoms von den anderen Leukosen abzugrenzen, scheint uns nicht sicher begründet, da die beiden mitgeteilten Krankengeschichten die Diagnose eines hypereosinophilen Syndroms keineswegs ausschließen. Über zytochemische Untersuchungen, durch die in fraglichen Fällen eventuell eine EL vom hypereosinophilen Syndrom abgegrenzt werden kann, berichteten kürzlich ACKERMAN (1) sowie OEHME *et al* (18)

Im klinischen Verlauf unterscheidet sich die akute EL nicht von anderen akuten Leukosen In den uns bekannten Beobachtungen war es ebenfalls unmöglich, die Zellproliferation durch Corticoide oder Cytostatica zu beeinflussen im Gegensatz zu dem i.a. guten Ansprechen der chronischen EL auf Corticoide (15) Purinethol (18) oder P^{32} (14) Wie in den anderen Beschreibungen einer akuten EL war es auch bei unserem Patienten zu ausgedehnten Infiltrationen in der Leber der Milz, den Nieren, den Lymphknoten

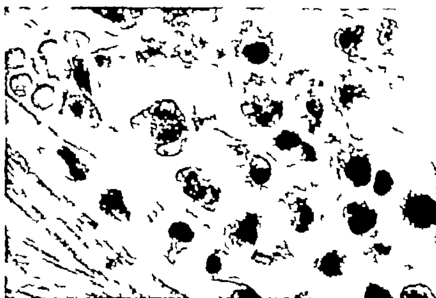


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Pathologisch-anatomische Diagnose Eosinophilen-Leukämie mit Infiltration von Milz, Leber Lymphknoten, Nieren und Herz. Bronchopneumonie von agranulozytärem Charakter

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Table III
Blood typing results in D. S. M.'s family

	Systems					
	ABO	MN	Rh	I	Sec. ABH	Sec. Le
Father	A_1B	MN	CCDee (R_1R_1)	/	Sec.	Sec.
Proposita (D. S. M.)	A_1B mod.	MN	CcDEe (R_1R_2 mod. or R_1R_2)	I	Sec.	Sec.
Sibs						
Sc.	A_1	N	CCDee (R_1R_1)	/	/	/
Le.	A_1	MN	CCDee (R_1R_1)	/	Sec.	Sec.
G.	A_1	N	CCDee (R_1R_1)	/	/	/
Su.	A_1	N	CCDee (R_1R_1)	/	/	/
N.	A_1	N	CCDee (R_1R_1)	/	/	/
J. M.	A_1B	N	CCDee ($R\ R_1$)	/	/	/
Lu.	B	N	CCDee (R_1R_1)	/	Sec.	Sec.
M.	A_2	MN	CCDee ($R\ R_1$)	/	/	/

The anti-Le^a was kindly furnished by Dr P. Levine. For information about other reagents an
the techniques used see the footnote of Table II

Table IV
Quantitative determinations of the degree of agglutination of D. S. M. blood and appropriate controls with anti-H, -A and A_1.

Antigens tested and date of determination (1965)	Percent of cells agglutinated	
	D. S. M.	Control
H or H-like		
August 27	99.7	99.9
September 3	99.8	99.9
September 17	99.9	99.9
September 27	99.9	99.9
October 27	78.5	99.6
A		
October 27	18.7	99.6
A		
October 27	17.9	99.6

According to the method of Filitti-Wurmser and Jacquot-Armand (2). The anti-H used was prepared in our laboratory from seed extracts of *Ulex europeus*, by the method of Boyd and Shapleigh (1). The anti-A and -A_1 were from commercial sources. The controls for the H determinations were 0 bloods; for the A and A_1 tests, an A_1 blood.

Table V

Some special tests performed in D. S. M.'s blood and saliva with appropriate controls

Antibodies	Titration after absorption with bloods						
	D. S. M. (1)	D. S. M. (2)	O (3)	A	B (3)	C−	C+
Anti-A	1:2	/	1:16	—	/	/	/
Anti-B	1:4	1:8	1:16	/	—	/	/
Anti-C	1:2	/	/	/	/	1:4	—
Tested against bloods	**Titration of the eluats from bloods**						
	D. S. M. (1)	D. S. M. (2)	O (3)	A	B (3)	C−	C+
A	1:2	/	—	1:1	/	/	/
B	1:2	1:4	—	/	1:1	/	/
C+	Undil.	/	/	/	/	—	Undil.
Date (1965)	**Inhibition titer of D. S. M.'s saliva with anti**						
	A	B	H				
August 31	1:2	Undil.	1:1				
September 7	1:1	Undil.	1:2				
Controls (D. S. M.'s father A_2B)	Undil.	1:2	1:2				
A_1B	1:8	1:2	1:1				

(1) Tested for A and B on September 4 and for C on September 21. (2) Tested on October 28. (3) Titration done simultaneously on two different occasions with identical results.

Table IV shows results of quantitative determinations of the degree of agglutination of D. S. M.'s blood and appropriate controls with anti H, A and A_1, as determined by the cell counting method described by Filitti Wurmser and Jacquot Armand (2). They indicate that D. S. M.'s blood behaved as a typical 0 blood in relation to the amount of H or H like substance on four different occasions and that the appearance of the A_1 substance at the final stage of her disease occurred in association with a corresponding decrease in the H or H-like material.

Table V presents additional tests performed in D. S. M.'s blood and appropriate controls. They show that the patient's blood absorbed and eluted A, B and C antibodies on occasions when her blood showed negative reactions with them and that there was some indication that the amount of B substance present in D. S. M.'s saliva may have also diminished, since on two different occasions they inhibited anti-B only undiluted.

Discussion

It should be clear at the outset that the many transfusions that patients afflicted with acute leukaemia receive, seriously hamper any attempt at clarifying the nature of the changes which have occurred in their blood. In our case, however the influence of the transfusions received could not explain many of the modifications observed. For instance, it is clear from the absorption and elution experiments that the patient's blood effectively had transformed A and B substances, which could be differentiated from the 0 cells she received from transfusions. In the changes related to the Rh system the influence of the transfusions is much more difficult to assess and at the present time we are inclined to regard the presence of an E-like antigen in her cells as being at least in part due to blood of transfusional origin (in the white population of Pôrto Alegre about 34 / of the Rh+ individuals are E+ see 9)

As far as we know only two other papers were published (5 6) in which changes in the blood of AB leukaemic individuals are reported In one (6) the patient was originally group AB and lost the B specificity in the terminal stage of her chronic lymphatic leukaemia. In the other study (5) the red cells of a patient suffering from eosinophilic leukaemia, and known from family studies to be of genotype A B were only partially agglutinated by anti-A and anti-B The abnormality in the A specificity was independent of the abnormality in the B specificity and the patient's blood was shown to contain A_2B, A_2, B and 0 cells but no A_1B or A_1 cells. In addition to these reports, DUNSFORD informed personally HOOGSTRATEN *et al.* (3) that he had observed loss of erythrocytic A antigen in another AB patient with leukaemia. Our case is therefore different of those reported in the sense that both the A and B activities have been lost. The partial reversion of A_1 at the end of the disease is interesting because it indicates that as in (5) the A and B abnormalities were probably independent it also shows that reversions can occur without clinical remissions.

Other points of interest in relation to the ABO changes are (a) The fact that the A_1 reversion occurred at the expenses of H like substance similar results were obtained by HOOGSTRATEN *et al.* (3) in a patient with acute myeloblastic leukaemia whose blood type A changed to Ag and in the terminal phase of his leukaemia reverted to subtype A_1 (b) No abnormality was observed in the

single determination we could perform of the I antigen this observation is of interest in view of the apparent relationship between the I and ABO systems (4) (c) The possible lowering of the level of B substance in D S M s saliva is also curious unfortunately we could not investigate this problem with more refined tests. The acceptance of this change as real would imply a more general type of abnormality in the synthesis of the B substance in leukaemics than is currently accepted. However it should be mentioned that VULCHANOV (10) reported recently a generalized loss of antigens in a case of chronic myeloid leukaemia.

The Rh abnormalities are difficult to interpret. It is clear however that the C changes can only be explained through abnormalities induced in some way by the disease process. D S M. s father is homozygous *CC* and she therefore should also have had at least one *C* gene. The appearance of the C substance in later tests is an additional fact in favor of this view More difficult to explain is the presence of a c-like substance which does not appear either in the father or sibs of the *proposita*. It could be of transfusional origin but the strength of the reactions make this explanation unlikely We favor the hypothesis that this c like activity may be due to the presence of the C substance, modified in some way The lowering of the c+ reaction strength with the appearance of C+ cells is in keeping with this view The fact that the c+ agglutination did not disappear completely speaks, however against it. Alternatively it could be thought that the E like activity would represent the transformed C substance, the c like reactions being due to transfusion blood. The obvious possibility that D S M. s mother is R_1R_2 can also be invoked but the fact that only D S M. and none of her eight sibs would have received an R^1 gene is against this hypothesis. The probability of such segregation is very low (1/256) Any way the importance of family studies to detect changes in the Rh system in leukaemic patients is clear and additional investigations in this direction may prove to be rewarding

Acknowledgements. Thanks are due to Dr MARIO B. CRUZ for referring the case to us, providing the results of the physical examination he performed in D S. M. in July of 1965 and for his general advice. Mr GUILHERME V SANTOS helped in the field investigations and Lic MARIA C. MALLMANN in some of the laboratory tests. Drs. H. FUDENBERG and P LEVINE furnished the anti-I and anti-Le[a] reagents. One of us (M. A.) is on leave of absence from the Departamento de Puericultura Pediatria da Faculdade de Medicina da Universidade Federal do Pará, with post-graduated fellowship provided by the University This work has been supported in part by the Rockefeller Foundation, Con-

selho Nacional de Pesquisas, Conselho de Pesquisas of the University of Rio Grande do Sul and PHS research grant RG-06238 from the Division of General Medical Sciences, Public Health Service (USA)

Summary

Blood and saliva studies are reported from a 13-year-old white Brazilian girl with acute myeloblastic leukaemia and her family. The patient developed changes in at least three blood group substances: the A and B antigens of the ABO system and the C antigen of the Rh blood group. The A and C changes showed reversions at the end of the disease process.

Zusammenfassung

Die vorliegende Arbeit beschreibt Untersuchungen an Blut und Speichel bei einem brasilianischen Mädchen von 13 Jahren mit akuter Myeloblasten-Leukämie. Die Untersuchungen wurden auch in der Familie dieses Mädchens gemacht. Die Patientin zeigte Veränderungen von wenigstens drei Blutgruppensubstanzen: den Antigenen A und B des Systems ABO und dem Antigen C der Blutgruppe Rh. Die Veränderungen von A und C zeigten eine Rückbildung am Ende der Krankheit.

Résumé

Les examens du sang et de la salive faits chez une enfant brésilienne de race blanche, âgée de treize ans, atteinte de leucémie myéloblastique aiguë, et auxquels l'on aussi procédé dans sa famille, font l'objet de ce travail. La malade a montré des altérations pour le moins de trois substances de groupes sanguins: soit des antigènes A et B du système ABO et de l'antigène C du groupe sanguin Rh. Les altérations des antigènes A et C régressèrent vers la fin de la maladie.

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Platelet Transfusion Therapy

A motion picture demonstrating a medical technique that is substantially reducing leukemia deaths due to hemorrhage has been released by the Acute Leukemia Task Force of the National Cancer Institute. The film, titled "Technique of Platelet Transfusion Therapy" was prepared as orientation material for blood bank personnel by the Task Force at the National Institutes of Health, US Public Health Service. The film shows the method of obtaining blood platelets by plasmapheresis, a procedure in which platelets and plasma are removed from an ordinary blood donation by centrifugation and a simple pressure device. The donor' red cells are immediately returned to him, so that a normal person can donate platelets from 2 units of blood as often as twice a week without ill effects. The platelets thus obtained, when given in adequate amounts, prevent the hemorrhages common among acute leukemia patients, and are a contributing factor to lengthening their life expectancy

The film presents step-by-step demonstration by a hematology technician of the platelet separation process, and portrays a family's role in contributing to leukemic child's well-being through platelet transfusions. The 16-millimeter color production, running time 21 75 min, was made with assistance from the American Red Cross, the District of Columbia General Hospital, and the Clinical Center of the National Institutes of Health. Requests to borrow the film without charge may be addressed to the US Public Health Service Audiovisual Facility Atlanta, Georgia 30333.

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Clinique médicale universitaire, Lausanne (Directeur: Prof. A. Vannotti)

Effet de l'insuline et de quelques autres facteurs sur la glycolyse des leucocytes humains mesurée in vitro

J. A. Antonioli, J. P. Felber et A. Vannotti

Parmi les tissus des mammifères, certains réagissent à l'insuline (tissu adipeux, cartilage, foie, glande mammaire, muscle, peau, utérus, vésicules séminales) alors que d'autres ne répondent pas à cette hormone (cerveau, aorte, intestin, tissu lymphoïde); certains tissus, enfin, ont donné lieu à des résultats expérimentaux apparemment contradictoires (cristallin, rétine, rein, hypophyse; 17). Il semble établi que les globules blancs de diabétiques présentent certaines altérations métaboliques, bien que la nature exacte de ces altérations soit encore discutée.

Le plus souvent, on décrit une diminution de la consommation de glucose (19, 6, 24), alors que la production d'acide lactique était normale (6) ou légèrement abaissée (19). Kalant et Schucher (16) observent une diminution de la consommation de glucose chez les leucocytes de diabétiques que lorsqu'ils sont incubés dans un milieu contenant du bicarbonate. Frei et al. (12) ont rapporté une baisse de l'activité de certaines enzymes glycolytiques chez les globules blancs de diabétiques. Alors que Valentine et al. (23) observaient une teneur normale en glycogène chez les granulocytes de diabétiques, Esmann (7), au contraire, enregistrait une diminution de 25% de cette teneur. Selon Esmann (8), il faut tenir compte d'un effet de foule ('crowding effect') lorsque l'on mesure l'activité métabolique des leucocytes. En effet, il est possible que l'activité moyenne d'une cellule diminue lorsque la concentration de cette cellule s'élève dans le milieu d'incubation. Esmann (10) trouve un tel effet de foule pour certaines activités métaboliques chez les granulocytes de sujets non diabétiques, alors que cet effet ne se manifeste pas chez les granulocytes de diabétiques; en tenant compte de l'effet de foule et en interpolant les activités métaboliques à de faibles concentrations cellulaires, il parvient à objectiver chez les granulocytes de diabétiques une diminution de la consommation de glucose, de la synthèse de glycogène et de la production de lactate. Toujours selon cet auteur (8), les polynucléaires de diabétiques sont le siège d'une lésion enzymatique au niveau de la déshydrogénase de l'acide lactique. Bybee et Rogers (3) ont montré que les leucocytes de diabétiques avaient un pouvoir de phagocytose amoindri, et que cette diminution dépendait non du sérum mais des cellules elles-mêmes. Raso (20) pas trouvé de différences entre la glycolyse de leucocytes d'exsudats péritonéaux de rats alloxanisés et celle de cellules provenant d'exsudats de rats normaux.

Selon ESMANN (7) l'administration d'insuline à un diabétique régularise, après un délai de 24 h, le métabolisme de ses leucocytes; cette constatation explique que l'on puisse observer un métabolisme leucocytaire normal chez des globules blancs de diabétiques traités. En revanche l'éventualité d'une action *in vitro* de l'insuline sur le métabolisme leucocytaire demeure sujet à controverse. Pour MARTIN *et al.* (19) l'insuline (0,1 U/ml) n'agit pas sur les leucocytes de sujets normaux, mais elle normalise le métabolisme des leucocytes de diabétiques. Selon DUMM (6) l'insuline (0,5 U/ml) augmente la consommation de glucose, mais n'affecte pas la production de lactate, chez les globules blancs de sujets normaux et de sujets diabétiques. Pour WEISBERGER et FIELD (24) l'insuline (0,6 U/ml) stimule la consommation de glucose et la production de lactate chez les leucocytes de sujets normaux et de sujets diabétiques. KALANT et SCHUCHER (16) ont trouvé que l'insuline (0,1 U/ml) augmentait légèrement la consommation de glucose des leucocytes des sujets normaux et fortement celle des leucocytes de diabétiques, à condition que le milieu d'incubation contienne du bicarbonate. Selon ESMANN (9) l'insuline à concentration très élevée (1,0 U/ml) et au cours d'une incubation de longue durée (4 h) stimule de manière peu accentuée la consommation de glucose et la production de lactate mais non la synthèse de glycogène, chez les leucocytes de sujets normaux; cet effet ne paraît pas être spécifique. Pour RAMO (20) l'insuline (2-10 U/ml) n'affecte pas la consommation de glucose des polynucléaires d'exsudats péritonéaux de rats normaux ou de rats alloxaniés.

La signification des résultats rapportés dans la littérature dépend étroitement des circonstances expérimentales au cours desquelles ils ont été obtenus et de la nature des dosages effectués. L'insuline exogène a été le plus souvent ajoutée à des concentrations très élevées, représentant environ 10 000 à 100 000 fois le taux d'insulinémie physiologique à jeun. On sait que les neutrophiles ne convertissent pas tout le glucose qu'ils prélèvent en acide lactique et que l'acide lactique formé ne provient pas nécessairement du glucose consommé. Si l'on dose simultanément la consommation du glucose, la production d'acide lactique et les variations de la teneur en glycogène, on remarque que les variations d'un de ces 3 paramètres métaboliques ne renseignent pas obligatoirement sur le comportement des 2 autres paramètres (1, 3). Nous savons, de plus, que le métabolisme des leucocytes exsudés est différent de celui des leucocytes circulants (1); les résultats obtenus avec des leucocytes exsudés ne peuvent donc être sans autre rapportés aux leucocytes circulants.

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unité cellulaire diminuent lorsque la concentration leucocytaire dans le milieu passe de 8 à 40×10^6 leucocytes par ml.

L activité métabolique peut également dépendre en partie de la concentration du glucose dans le milieu d'incubation. Une corrélation de ce type pourrait être un important facteur de régulation du métabolisme leucocytaire in vivo notamment lors de diabète et de l'hyperglycémie qui l'accompagne, et dans les exsudats où le taux de glucose est très variable. Selon Glover et al. (13) McKinney et al. (18) et Martin et al. (19) la production de lactate par les leucocytes est optimale pour une concentration de glucose de 5,6 mM; au-dessous et au-dessus de cette concentration, la production de lactate est moindre. Selon Dubois (6) la variation de la concentration de glucose dans le milieu entre 4 1 et 19 mM influence ni la consommation du glucose, ni la production du lactate.

Les leucocytes sont capables de lier très activement de l'insuline marquée au S^{35} (14) ou au I^{131} (14 22) Bien que ce pouvoir de liaison ne soit pas différent chez les leucocytes de diabétiques de ce qu'il est chez ceux de sujets non diabétiques (22) on peut néanmoins envisager l'hypothèse selon laquelle le métabolisme leucocytaire serait fonction de la quantité d'insuline endogène liée aux cellules antérieurement à leur pré lèvement. Si tel était le cas, on pourrait attendre à ce que le métabolisme des leucocytes varie chez un même sujet selon qu'il est à jeun ou dans une phase post-prandiale; on pourrait attendre également à ce qu une réponse à de l'insuline exogène soit observée de préférence chez des leucocytes de sujets à jeun. Selon Dubois (6) cependant, la glycolyse leucocytaire est semblable chez des cellules de sujets à jeun et chez celles de sujets dans une phase post-prandiale.

Enfin, nous avons vu que les globules blancs suspendus dans du sérum consommaient davantage de glucose et produisaient davantage d acide lactique que ceux suspendus dans un milieu KGAG (2) On peut se demander notamment si cet effet est dû à la présence d'insuline endogène ou si l'insuline endogène n agit qu en présence de facteurs sériques.

Nous avons entrepris ce travail afin de déterminer l influence de la concentration cellulaire et de la concentration du glucose sur la glycolyse des leucocytes humains. Nous avons également voulu comparer le métabolisme des leucocytes prélevés chez un sujet à jeun à celui de ces mêmes cellules prélevées chez ce même sujet lors d une épreuve d'hyperglycémie provoquée, en dosant l'in sulinémie qui régnait au moment du prélèvement des cellules. Enfin nous avons voulu voir si l effet des doses d'insuline exogène proches des physiologiques pouvait dépendre de l'insulinémie du sujet au moment du prélèvement des cellules ou de la présence de sérum dans le milieu d'incubation

Matériel et Méthodes

Le sang est prélevé par ponction veineuse chez des sujets indemnes d'affections métaboliques, d'infections ou de maladies hématologiques. L'isolation des leucocytes se fait selon la technique habituelle, sauf que chez l'homme la sédimentation des érythro-

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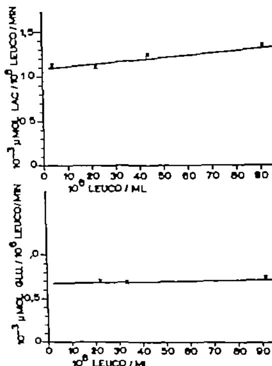


Fig 1 Effet de la concentration des leucocytes dans le milieu sur l'activité glycolytique aérobie des suspensions. Chaque point représente la moyenne de 2 ou 3 expériences. La concentration du glucose dans le milieu est de 5,6 mM. La droite de régression de la production de lactate en fonction de la concentration cellulaire est donnée par l'équation

$$Y = 1{,}077 + 0{,}00264\ X\ (r_{yx} = 0{,}239\ ;\ n = 25)$$

La droite de régression de la consommation de glucose en fonction de la concentration cellulaire est donnée par l'équation

$$Y = 0{,}685 + 0{,}00024\ X\ (r_{yx} = 0{,}308\ ;\ n = 25)$$

synthèse de glycogène demeurent stables (le test F indique une probabilité P > 0 05 pour ces 2 activités) en revanche, la consommation de glucose est stable entre 2 74 et 5 70 mM (le test F indique une probabilité P > 0 05) mais elle augmente linéairement avec le logarithme de la concentration de glucose entre 5,70 et 15 78 mM de glucose dans le milieu (le test F indique une probabilité P < 0,01)

Effet de la glycémie et de l'insulinémie du sujet au moment de la prise de sang sur l'activité glycolytique aérobie des suspensions On a prélevé du sang veineux au temps 0 et au temps 45 min d une épreuve d'hyperglycémie provoquée chez 5 sujets différents à jeun. On a mesuré l'insulinémie et la glycémie dans les 2 échantillons de sang dont on

qu au temps 45 min (tableau I) Le défaut de réponse à l'insuline ne provient donc pas d une saturation des leucocytes par de l'in suline endogène avant leur prélèvement.

On a préparé 5 suspensions de leucocytes dans un milieu KGAG contenant 1 volume de sérum autologue pour 4 volumes de KGAG Chaque suspension a été divisée en 2 parties, dont 1 une a été en richie par l adjonction d insuline exogène (0 5 mU/ml) Cette ad jonction d'insuline (tableau II) ne modifie pas la consommation du glucose, mais stimule légèrement, quoique significativement, la production du lactate (le test t utilisé dans la méthode des couples indique une probabilité $P < 0\ 01$) Des essais pour augmenter cette stimulation en préincubant le mélange suspension + insuline, en remplaçant l insuline bovine par une dose équivalente d'insuline humaine ou en augmentant la concentration d insuline dans le milieu n ont pas réussi. Il semble donc qu en présence de sérum dilué, l on observe une stimulation non spécifique de la production de lactate par les suspensions.

Discussion

L examen des suspensions incubées à des concentrations va riables ne révèle aucun effet de foule au contraire, les activités métaboliques rapportées à l unité cellulaire tendent à augmenter à mesure que la concentration cellulaire croît. Ce phénomène nous paraît refléter davantage un artifice qu une modification métabolique réelle. En effet, les numérations cellulaires s effectuent dans les suspensions prêtes à l incubation le comptage au moyen du *Coulter Counter* favorise une erreur systématique par défaut lors des concentrations cellulaires élevées. Nos résultats se rapprochent de ceux obtenus par Glover *et al* (13) avec des leucocytes leucémiques et par Rabio et Conard (21) avec des polynucléaires exsudés de rat. En revanche, ils s opposent à ceux rapportés par Barron et Harrop (4) avec des leucocytes leucémiques et par Esmann (10) avec des leucocytes humains normaux Esmann en particulier a travaillé avec un système très proche du nôtre le mode d'isolation des globules blancs qu il utilise ne diffère que peu de celui que nous avons adopté, sauf qu'il ne comporte aucune hémolyse des érythrocytes dans le surnageant après sédimentation le milieu d Esmann est aussi un milieu KGAG mais il contient davantage de glucose (16,7 mM au lieu de 5 6 mM) il renferme de l acide ascorbique

et l'incubation dure plus longtemps (2 h au lieu de 1 h) Dans ces conditions, ESMANN trouve que la consommation de glucose et la production de lactate par unité cellulaire diminuent de 50 % lorsque la concentration cellulaire passe de 8 à 40×10⁶ leucocytes par ml. Voici en regard les activités métaboliques enregistrées par ESMANN (8) après transformation des données originales dans les mêmes unités que les nôtres et celles que nous avons trouvées dans l expérience sur l effet de la concentration du glucose lors de l'incubation en présence de 15 78 mM de glucose qui est la concentration de glucose la plus proche de celle d ESMANN (8, 10)

activité métabolique	ESMANN	ANTONIOLI	unités
teneur en glycogène	15	15 7	µg/10⁶ granulocytes
ynthèse de glycogène	0,19	0,386	10⁻³ µmol/10⁶ leucocytes/mm
consommation de glucose	1 02	1,289	
production de lactate	1 93	1,321	

Alors que la teneur en glycogène et la consommation de glucose sont très voisines dans les 2 cas, les suspensions d ESMANN produisent davantage de lactate tandis que les nôtres synthétisent davantage de glycogène. Il faut probablement rechercher l explication de l effet de foule soit dans la durée de l'incubation, soit dans le taux élevé du glucose dans le milieu, soit dans une association de ces 2 facteurs.

L étude de l effet de la concentration du glucose dans le milieu sur l activité métabolique des suspensions est en accord avec les observations de GLOVER *et al* (13) mais est en désaccord avec celles de MCKINNEY *et al.* (18) MARTIN *et al* (19) DUBOIS (6) et ESMANN (8) Dans notre système il semble y avoir dissociation entre l entrée du glucose dans la cellule, qui dépend partiellement de la concentration du glucose dans le milieu, et la transformation de ce glucose par le métabolisme cellulaire, qui est indépendant de la concentration du glucose dans le milieu. Il est peu probable que le glucose consommé soit oxydé en plus grande quantité lors d incubations en présence de concentrations élevées de ce sucre en effet, en raison de l'effet CRABTREE, qui a été décrit chez les leucocytes notamment par MCKINNEY *et al* (18) la présence de glucose dans le milieu diminue la consommation d oxygène par les suspensions. On peut donc envisager l'hypothèse selon laquelle le glucose qui entre dans la cellule

s'accumule dans un premier temps sous forme de phosphates d'hexose. La prolongation de la durée de l'incubation 2 heures chez ESMANN (8) 2 à 2 ½ heures chez DUMM (6) 4 heures chez MCKINNEY *et al* (18) et chez MARTIN *et al* (19) peut masquer ce phénomène initial. A ce propos, ESMANN (8) remarque que la consommation de glucose, la production de lactate et la synthèse de glycogène qu'il observe dans ses suspensions sont plus élevées au cours de la première heure d'incubation qu'au cours de la seconde.

Le taux sanguin d'insuline endogène ne paraît pas orienter durablement le métabolisme des leucocytes avant leur prélèvement, ce qui confirme les premières constatations de DUMM (6) La stimulation du métabolisme leucocytaire par du sérum autologue dilué 1,25 fois (2) ne se retrouve que partiellement avec du sérum dilué 5 fois seule la production d'acide lactique est augmentée, mais cet accroissement est du même ordre (plus de 40%) avec les deux dilutions de sérum. Des expériences préliminaires ont montré que cette stimulation de la formation de lactate variait selon la nature du sérum cette stimulation est plus marquée avec du sérum prélevé au temps 60 d'une épreuve d'hyperglycémie provoquée qu'avec du sérum prélevé au temps 0 d'une telle épreuve. Ces constatations permettent d'envisager l'hypothèse d'un facteur sérique, probablement lié au métabolisme général, et qui agirait davantage sur l'utilisation du glucose par les leucocytes que sur l'entrée de ce sucre dans les cellules.

L'insuline exogène, à une concentration qui représente 100 à 200 fois l'insulinémie normale à jeun, n'agit pas, dans nos conditions d'expérience sur le métabolisme leucocytaire. Cette absence d'effet ne peut pas se rattacher à un défaut de bicarbonate dans le milieu d'incubation (16) elle ne peut pas davantage être expliquée par une orientation préalable du métabolisme leucocytaire par de l'insuline endogène, ni par le manque d'un facteur sérique indispensable à son action. La légère stimulation de la production d'acide lactique observée lors de l'adjonction d'insuline dans le milieu contenant du sérum autologue dilué 5 fois ne paraît pas spécifique En effet, on n'observe aucun accroissement de la consommation de glucose, et divers essais tendant à augmenter cette stimulation en préincubant le sérum et l'insuline en variant les concentrations d'insuline, en remplaçant l'insuline bovine par de l'insuline humaine, ont échoué. Il faut remarquer que la consommation de glucose par les leucocytes est considérable elle dépasse de beaucoup

celle d un tissu tel que le diaphragme chez lequel l'insuline contrôle l entrée du glucose. DUNN (6) rapporte que le diaphragme de rat consomme 4 à 6 mg de glucose par g de poids humide par heure et compare cette consommation à celle des leucocytes humains. En nous référant à la consommation de glucose par les polynucléaires de rat telle que l observent RAMO et CONARD (21) et en admettant que 10^7 leucocytes ont un poids humide de 6 mg (6) ou que 5 g de poids humide équivalent à 1 g de poids sec (8) on trouve que cette consommation est de l ordre de 15 à 38 mg de glucose par g de poids humide par heure même si l on admet que les granulocytes exsudés consomment davantage de glucose que leurs homologues circulants (1) la différence entre leucocytes et diaphragme demeure importante Cependant, le métabolisme leucocytaire est sensible à une régulation endocrinienne l administration in vivo de cortisone stimule, après quelques heures, la consommation de glucose, la synthèse de glycogène et la production d acide lactique (3)

Remerciements Nous tenons tout particulièrement à remercier ici le Fonds National pour la Recherche Scientifique pour son appui financier le Centre Romand de Transfusion Sanguine pour son concours dans l'obtention d'échantillons de sangs de sujets normaux, et Mlle ODILE LEVRAT pour son assistance.

Résumé

On prépare des suspensions de leucocytes humains de sujets non diabétiques et on étudie l'effet de divers facteurs sur leur métabolisme aérobie *in vitro* On ne trouve pas d'effet de foule en ce qui concerne la consommation d glucose et la production d'acide lactique. En l'absence de glucose exogène, on observe une formation notable d'acide lactique et une diminution de la teneur en glycogène intra-cellulaire lorsque le milieu contient du glucose, la production d'acide lactique augmente et la teneur en glycogène intra-cellulaire élève mais elles ne varient pas en fonction de la concentration extra-cellulaire de glucose. La consommation de glucose en revanche, dépend dans une certaine mesure de la concentration extra-cellulaire de glucose La consommation de glucose et la production de lactate sont semblables chez des leucocytes de sujets à jeun et chez des leucocytes de sujets en état d'hyperglycémie et d'hyperinsulinémie. La présence dans le milieu de sérum autologue stimule fortement la production de lactate sans affecter la consommation de glucose. *In vitro* la consommation de glucose et la production d'acide lactique ne sont pas modifiées par l'adjonction d'insuline Ces résultats sont discutés en fonction du contrôle du métabolisme leucocytaire.

Summary

Leucocyte suspensions were prepared in human non-diabetic subjects and their aerobic metabolic activity measured *in vitro* under various conditions. No crowding effect was noted in the glucose consumption or in the lactic acid production. I absence

of added glucose, lactic acid was produced and intracellular glycogen diminished when the medium contained glucose the lactic acid formation increased and intracellular glycogen was synthesized *this lactic* acid production and glycogen synthesis did not vary as function of the glucose concentration in the medium. On the other hand, the glucose consumption depends in certain extent on the glucose concentration in the medium. Leucocytes from fasting subjects and leucocytes from subjects in *state of hyperglycemia and hyperinsulinaemia* showed the same glucose consumption and the same lactic acid production. In medium containing autologue serum, the lactic acid production, but not the glucose consumption, was strongly stimulated. *In vitro* neither the glucose consumption, nor the lactic acid production were affected by addition of insulin. These results are discussed in relation to the regulation of the leucocyte metabolism.

Zusammenfassung

An Suspensionen menschlicher Leukozyten von Nichtdiabetikern wird die Wirkung verschiedener Faktoren auf den aeroben Stoffwechsel in vitro untersucht. Mit Bezug auf den Glukoseverbrauch und die Milchsäureproduktion lässt sich keine Massenwirkung feststellen. Bei Fehlen exogener Glukose lassen sich eine beträchtliche Milchsäurebildung und eine Verminderung des intrazellulären Glykogengehaltes nachweisen. Wenn das Milieu Glukose enthält, nehmen Milchsäureproduktion und intrazellulärer Glykogengehalt zu, sie zeigen jedoch keine Veränderungen in Abhängigkeit von der extrazellulären Glukosekonzentration. Bei nüchternen Individuen sind Glukoseverbrauch und Milchsäureproduktion gleich wie bei Individuen mit Hyperglykämie und *mit Hyperinsulinämie. Die Gegenwart von autologem Serum im Milieu steigert die* Milchsäureproduktion stark, ohne den Glukoseverbrauch zu beeinflussen. In vitro werden Glukoseverbrauch und Milchsäureproduktion durch Zugabe von Insulin nicht beeinflußt. Die Ergebnisse werden im Hinblick auf die Regulation des Leukozytenstoffwechsels diskutiert.

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Haemoglobin Koelliker: A New Acquired Haemoglobin Appearing after Severe Haemolysis: α_2 minus 141 Arg β_2

H. R. MARTI, D. BEALE and H. LEHMANN

Haemoglobin Koelliker was detected in a case of paroxysmal nocturnal haemoglobinuria (Fig 1) and subsequently found in other patients with acute haemolysis of different origin (6). In a previous paper the new haemoglobin was described to differ from normal haemoglobin A at the C-terminal end of the α-polypeptide chains, the arginine 141 being missing (7). The following report completes these results by final amino acid analyses.

Case Reports

The hitherto unknown fast moving haemoglobin fraction was first demonstrated in 3 patients with haemolysis of different origin.

(1) A 29 year old female with *paroxysmal nocturnal haemoglobinuria* shows normal haemoglobin pattern of the erythrocytes except slight increase in HbF. HbF 2.1% (norm. <0.8%), HbA_2 1.9%, no abnormal haemoglobin is detectable. The activities of the red cell enzymes glucose-6-phosphate dehydrogenase, pyruvate kinase, glutathione reductase and the amount of reduced glutathione are within the normal range. At the time of each heamolytic crisis starch block electrophoresis with plasma and urine at pH 8.6 with subsequent benzidine staining shows 3 fractions: normal free HbA_1, methaemalbumin, and fraction moving slightly faster than Hb A_1. The latter one is usually more pronounced in the urine than in plasma or serum. Haptoglobin is absent.

(2) By courtesy of Prof. DACIE, London, we received serum sample from an adult male with *haemolytic anaemia following cardiac operation*. The patient was operated three months previously and valve prosthesis was placed in the aortic valve region. W found secondary ahaptoglobinaemia and with starch block electrophoresis two benzidine positive fractions, one with the mobility of methaemalbumin and the other migrating slightly faster than Hb A_1. N normal free Hb A_1 was present.

(3) I *blood sample partially deteriorated* during transport from an adult female patient with haemolytic crisis of unknown origin similar fraction was demonstrable in the haemolysat corresponding to 37 of the total haemoglobin. At pH 6.5 this fraction migrated more slowly toward the cathode than Hb A_1. After the haemolytic crisis no abnormal haemoglobin was detectable in erythrocytes and serum.

(4) In *fresh artificial mixtures of normal haemolysate with normal plasma* single haemoglobin fraction is demonstrable with the mobility of Hb A_1. But after storage 37 C

during 24 h or at +4 °C during 10 days fast moving haemoglobin fraction appears with the mobility as described above.

In all the instances mentioned the same fractions are obtained when the haemoglobin is converted to Met-Hb, CN Met-Hb and CO-Hb.

Material and Methods

Urine of the first propositus, the 29-year-old female with paroxysmal nocturnal haemoglobinuria, and erythrocytes of normal adult blood donors were used. Haemoglobin A solution were prepared by haemolysis of washed erythrocytes in the presence of carbontetrachloride. Haemoglobin Koelliker was prepared from urine as follows The urine was dialysed with acuum at the outside of the membrane. The resulting concentrated mixture of macromolecules contained haemoglobin A, haemoglobin Koelliker and other proteins. This mixture was submitted to paper electrophoresis using TRIS buffer pH 8.9 and the part of the paper containing the Koelliker fraction was cut out and eluted. The eluate was concentrated in vacuo and paper electrophoresis with subsequent elution and concentration were repeated until on electrophoresis only single band of haemoglobin was seen. The final sample was then incubated for 2 h 37 °C with ammonium sulphate, final concentration 55%, to precipitate any globulins with the electrophoretic mobility of haemoglobin Koelliker After centrifuging, the supernatant was dialysed against water to remove the ammonium sulphate, and concentrated in vacuo

Fingerprints of purified haemoglobin were prepared according to Ingram (3) and Baglioni (2). A detailed description *of the procedures used in our laboratory* with full references, was given in the paper by Wittmann Williams *et al.* (11)

Results

On comparing the fingerprints of haemoglobin Koelliker with those of normal adult haemoglobin A, it was noted that the peptide αTpXIV (140 Tyr 141Arg) was missing but no additional peptide could be found, except for a faint spot in the neutral area which gave colour reactions for tyrosine and tryptophan (Fig 2) This spot was probably the result of a partial chymotryptic break at β 37 tryptophan due to slight chymotryptic activity of the trypsin sample and represented β 31-37 (Leu Leu-Val-Val Tyr Pro-Try) When highly purified trypsin samples were used or chymotryptic activity was inhibited with L (1 tosylamido-2 phenyl)ethyl-chloromethyl ketone (4) the additional spot was not obtained. To find out what had happened to the missing peptide the amino acid analysis (10) of globin from haemoglobin Koelliker was compared with that of globin from normal adult haemoglobin A. This showed that only one arginine residue was missing in haemoglobin Koelliker (Table) and that the tyrosine content of haemoglobin A and haemoglobin Koelliker was the same.

Table

Amino acid analysis of globin from haemoglobin Koelliker and haemoglobin A.

	Globin Koelliker			*Globin A*			
	24 h hydrolysis	48 h hydrolysis	corrected residues	24 h hydrolysis	48 h hydrolysis	corrected residues	Expected residues
Asp	24.2	24.9	24—25	23.8	24.6	24—25	25
Thr*	15.5	14.8	16	15.3	14.9	16	16
Ser*	13.4	15.0	16	13.5	15.2	16	16
Glu	15.6	15.9	16	16.0	16.2	16	16
Pro	14.0	14.5	14	14.3	14.4	14	14
Gly	19.3	20.4	19—20	19.8	20.3	20	20
Ala	34.9	33.6	35—36	35.2	36.4	35—36	36
Cys	+	+	+	+	+	+	3
Val	30.0	30.4	30	29.1	29.9	29—30	31
Met	2.7	2.5	3	2.8	2.5	3	3
Leu	33.3	35.9	35—36	35.8	36.3	36	36
Tyr*	5.7	5.2	6	5.6	5.2	6	6
Phe	14.5	15.2	15	14.3	14.8	14—15	15
Lys	22.1	21.4	21—22	22.4	21.6	22	22
His	18.6	18.2	18—19	18.7	18.4	18—19	19
Arg	4.8	4.9	5	5.6	5.9	6	6

* corrected for loss on hydrolysis

Tryptophan was not measured, and cysteine was demonstrated but not quantitated

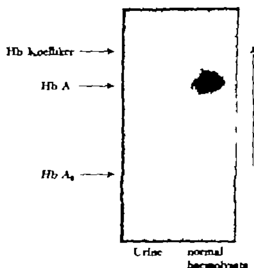


Fig. 1 Haemoglobin Koelliker in the urine in paroxysmal nocturnal haemoglobinuria. Starch block electrophoresis pH 8.6.

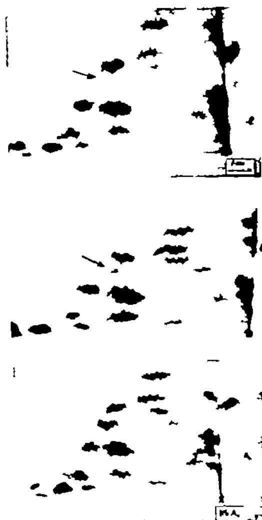


Fig [illegible]. Fingerprint analysis of haemoglobin Koelliker from urine (above) and artificially in vitro produced haemoglobin Koelliker (middle) compared with normal haemoglobin A (below). The arrow indicates the place of the missing α TpXIV spot.

Treatment of the globin from haemoglobin Koelliker with carboxypeptidase A + B (1) released 1 mol histidine and 2 moles tyrosine but no arginine. Treatment of globin from haemoglobin A, on the other hand, released 1 mol histidine, 2 moles tyrosine and 1 mol arginine.

These results suggest that haemoglobin Koelliker differs from haemoglobin A by the absence of the C-terminal arginine residue of the α-chain.

To examine whether haemoglobin Koelliker could be a degradation product of haemoglobin A, a solution of haemoglobin A was incubated with fresh human serum for 2 h at 37 C. To remove the serum proteins, the serum was then incubated over night with ammonium sulphate, final concentration 55%. The precipitate was removed by centrifuging the supernatant was concentrated *in vacuo* to the original volume and again incubated with ammonium sulphate. This procedure was repeated until no further precipitate appeared. The haemoglobin solution was then submitted to paper electrophoresis. Besides haemoglobin A another haemoglobin could be seen with a mobility which was indistinguishable from that of the haemoglobin Koelliker prepared from urine. This fraction was eluted and purified by repeated electrophoresis. Fingerprints of this haemoglobin were identical with those from haemoglobin Koelliker (Fig 2)

Discussion

Small amounts of free haemoglobin in the plasma are specifically bound to haptoglobin. Other serum proteins, migrating in the range of the α_2 and β-globulins, but different from haptoglobin have also been found to bind some haemoglobin or myoglobin (8, 12) If the level of free haemoglobin or myoglobin exceeds the binding capacity of these plasma proteins, both pigments are excreted by the kidney The low molecular weight, being about 68000 for haemoglobin and 17000 for myoglobin, allows the free passage of the molecules through the glomerular membrane into the urine In myoglobinuria the myoglobin in the urine was never found to differ in any way from muscle myoglobin, and both have the same electrophoretic migration rate (5 9) In haemoglobinuria after severe haemolysis however we were able to demonstrate the presence of a hitherto unknown haemoglobin fraction migrating faster than Hb A in the electrophoresis at alkaline pH (6) The haemolytic urine contains predominantly this fast moving haemoglobin component and only small amounts of Hb A (Fig 1) The abnormal component was found to be present in the plasma after intravascular haemolysis of different origin and could be produced *in vitro* by incubation of normal plasma with normal haemoglobin for 24 h

at 37 C or for 10 days at 4 C. The possibility of a complex of haemoglobin bound to another protein could be ruled out by the finger print analysis (7) The present results produce the final proof that haemoglobin Koelliker differs from normal haemoglobin A by absence of the C-terminal arginine of the α-chains and can be denoted as $\alpha_2^{-141\,\mathrm{Arg}}\beta$ or $\alpha_2^{-141\,\mathrm{Arg}}\beta_2$.

The evidence points to haemoglobin Koelliker being formed by a plasma carboxypeptidase which is capable of removing the carboxyterminal arginine of the α-chain, but which is not able to detach the carboxyterminal histidine of the β-chain or to continue the degradation of the α-chain by removing the 140-tyrosine after the removal of the 141-arginine.

Whereas in mild haemolysis free haemoglobin would be bound and removed by the haptoglobins, a pronounced haemolysis would allow enough free haemoglobin to circulate for it to be attacked by this plasma carboxypeptidase, and then to appear as haemoglobin Koelliker in the urine.

Summary

Haemoglobin Koelliker is an electrophoretically fast moving haemoglobin which is formed *in vivo* and *in vitro* when free normal Hb A is present in normal plasma. It can be found in plasma and urine after severe haemolysis from different causes. The abnormality rests in the C-terminal end of the α-polypeptide chains. Haemoglobin Koelliker is haemoglobin A which has lost the C-terminal 141 arginine of the α-chains, probably by the action of carboxypeptidase present in normal plasma.

Zusammenfassung

Hämoglobin Koelliker ist ein elektrophoretisch schnell wanderndes Hämoglobin, das *in vivo* und *in vitro* auftritt, wenn freies Hämoglobin A in normalem Plasma gelöst ist. Es wird in Plasma und Urin nach schwerer Hämolyse irgendwelcher Genese angetroffen. Die anomale Komponente unterscheidet sich vom normalen Blutfarbstoff im C-terminalen Ende der α-Polypeptidketten. Hämoglobin Koelliker ist normales Hämoglobin A, welches das C-terminale Arginin 141 der α-Ketten verloren hat. Das C-terminale Arginin wird wahrscheinlich durch eine in normalem Plasma vorhandene Carboxypeptidase abgespalten.

Résumé

L'hémoglobine Koelliker est une hémoglobine qui une grande vitesse de migration électrophorétique et qui est formée *in vivo* et *in vitro* quand de l'hémoglobine libre est dissoute dans du plasma normal. Elle peut être trouvée dans le plasma et dans les urines après une forte hémolyse de cause quelconque. L'anomalie se trouve en position C-terminale de la chaîne polypeptidique alpha. L'hémoglobine Koelliker est une hémoglobine A qui perdu l'arginine 141 en position C-terminale de la chaîne alpha, probablement par l'action d'une carbopeptidase présente dans le plasma normal.

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The Thalassaemia Syndromes

VI Two Subtypes of Sickle Cell-Beta Thalassaemia Disease (a) Normocytic Type of Sickle Cell-Beta Thalassaemia Disease (b) Microcytic Type of Sickle Cell-Beta Thalassaemia Disease

M. AKSOY and S ERDEM

Since the first description of sickle cell thalassaemia disease by SILVESTRONI and BIANCO (1) this haemoglobinopathy was characterized haematologically by a hypochromic microcytic anaemia of variable severity (2–8) Therefore, microcytosis evident by morphologic examination of the blood films and the determinations of mean corpuscular volume has been considered one of the foremost features of this congenital haemolytic anaemia by several investigators (1 2 4 7–9) Indeed, this finding has been found in the large majority of the patients with sickle cell thalassaemia disease. Furthermore, the similarity of haematologic picture of sickle cell-thalassaemia disease and intermediate type of COOLEY's anaemia has been noted by several investigators (4 6)

On the other hand, AKSOY and LEHMANN and later AKSOY reported a total of 11 cases of this haemoglobinopathy among Eti-Turks (10 11) In four of these patients the anaemia was hypochromic microcytic and in the remaining it was normocytic. In these patients with sickle cell-thalassaemia disease the clinical picture and routine haematologic studies suggested sickle cell-anaemia rather than intermediate type of COOLEY's anaemia.

Recently on the basis of the genetic concept of INGRAM and STRETTON (12) concerning thalassaemia, sickle cell-thalassaemia disease has been classified into two major types.

(a) Sickle cell-alpha thalassaemia disease, non interacting or asymptomatic type, characterized by a mild clinical and haemato-

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logic picture normal amount of haemoglobins A_2 and F and rather low level of haemoglobin S fraction similar to the electrophoretic pattern of sickle cell trait.

(b) Sickle cell-beta thalassaemia disease, interacting type, characterized by a severe or moderately severe clinical and haematologic picture of this congenital haemolytic anaemia with increased levels of haemoglobin A_2 and/or haemoglobin F and high level of haemoglobin S fraction.

In the last few years, we were able to perform haemoglobin analysis with new techniques in one of our cases with sickle cell thalassaemia disease resembling sickle cell anaemia rather than intermediate type of COOLEY's anaemia. Therefore we thought that it was tempting to try to compare the examples of different types, namely normocytic and microcytic types, of this haemoglobinopathy

This report describes the haematologic and genetic studies of two patients with sickle cell beta thalassaemia disease. Although the clinical manifestations and the results of haemoglobin analyses were identical, the type of anaemia was different namely hypochromic and microcytic in one of the patients and normocytic in the other

Methods

The haematologic methods were all standard techniques. Mean corpuscular volume was estimated by using WINTROBE haematocrit according to the instructions of the same author (8) Haematocrit was centrifuged in Juan 57 Model apparatus, t 4000 r.p.m./30 min. Haemoglobin analyses were performed by starch gel electrophoresis using borate buffer at pH 8.6 according to the method of SMITHIES slightly modified (13, 14) Haemoglobin F was determined by the method of SINGER et al. (15). Quantitative estimations of haemoglobin A_2 were performed by the method DEAE-cellulose chromatography following the descriptions of HUISMAN and DOZY (16). As haemoglobin S can not be separated from haemoglobin A_2 by DEAE-cellulose chromatography starch gel electrophoresis technique was used when haemoglobin S was present in the blood of the persons investigated (14) I these methods normal values for haemoglobin A_2 range between 2 and 3 for DEAE-cellulose chromatography and 1.5 and 4% for starch gel electrophoresis (13, 14)

Case Reports

Case 1

A.A., 22 year old girl was brought to the Outpatient Department of Çapa Internal Clinic because of anaemia, pains in bone and joints and palpitation. She was followed up since 1955 and reported in 1957 by AKSOY and LEHMANN elsewhere in series of sickle cell-thalassaemia disease among Eti-Turks as case 4 (10) She became

Sephadex A 50 Medium anion exchanger Pharmacia Uppsala (Sweden).



Fig. 1. (*A*) Blood film of the patient with normocytic type of sickle cell-beta thalassaemia disease (case 1) demonstrating anisocytosis with numerous target-cells. (*B*) Blood film of the patient with microcytic type of sickle cell- beta thalassaemia disease (case 2) demonstrating marked anisocytosis and microcytosis with numerous target cells (May-Grünwald-Giemsa stain).

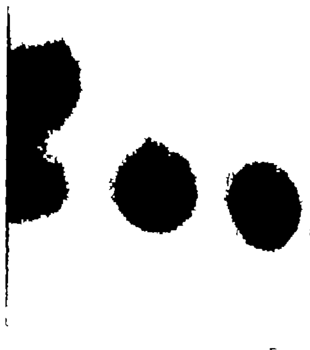


Fig. 2. Starch gel electrophoresis at borate buffer 0.025 M pH 8.6. From left to right $A+S+A_2$ (Sickle cell trait: Hb A 59.5, Hb S 37% and Hb A_2 3.5), $A+F+S+$ increased A_2 (sickle cell- beta thalassaemia disease, normocytic type: case 1: Hb A 5.9%, Hb F 8.4, Hb S 79.2% and Hb A_2 6.5), $F+S+A_2$ (sickle cell-anaemia: Hb F 6.5%, Hb S 89.7 and Hb A_2 3.8%). The gel was photographed without staining.

nearly invalide from severe abdominal pains and pains in bone and joints of several hours or days duration. Therefore, she was hospitalized often in the hospitals in Mersin and Istanbul.

Physical examination revealed an anaemic looking white girl with rather short stature. Her sclerae were subicteric and there was brown-yellow tint to the skin. The liver edge was palpable one and half finger-breadths below the right costal margin, but the spleen was only palpable. Mild frontal bossing was present. Pulse rate was between 88 and 140/min. A presystolic murmure was heard over apex and pulmonary area. The second pulmonary sound was accentuated. Blood pressure was 130/50 mm Hg. X-ray study of the chest disclosed mild left ventricular enlargement and prominence of the shadow of pulmonary artery (mitral configuration). In right anterior oblique position the left atrium was enlarged and there was mild compression on oesophagus. An electrocardiogram showed sinus tachycardia, P mitrale, marked ST depression in V4 to V6 and heart in semivertical position with marked clockwise rotation. Venous pressure 300 mm saline. Her haematologic findings are summarized in Table I.

Family study. The mother had sickle cell trait and the father was beta-thalassaemic heterozygote. The parents' haematologic findings are summarized in Table I.

Table I

Haematologic findings of two patients with normocytic and microcytic types of sickle cell-beta thalassaemia disease

Haematologic data	Case I	Mother	Father	Case II	Mother	Father
RBC (10^6/mm³)	3.40	4.70	4.90	3.40	4.10	4.60
Hb (g%)	8.9	13.5	1	7.3	11.3	13.6
WBC/mm³	9,200	8,000	7,000	10,800	7,200	6,500
Reticulocytes ( )	6.5	1	1.5	3.6	3	0.5
Haematocrit (%)	31.5	42	44	26	33	43
MCV (μ^3)	92.6	87	89	75	80	93
MCHb. ($\mu\mu g$)	26.2	27	24	22	27	29
NRC/100 WBC	4	0	0	1	0	0
Polychromatophilia	+	—	—	+	—	—
Poikilocytosis	—	—	—	+	—	—
Hypochromia	—	—	+	++	++	—
Microcytosis	—	—	—	++	++	—
Basophilic stippling	—	—	—	+	+	—
Target-cells	++++	—	+++	+++	++	—
Sickle-cells	+	—	—	—	—	—
Osmotic fragility (NaCl)	0.44–0.16	0.44–0.3	0.44–0.2	0.44–0.18	0.44–0.22	0.44–0.3
Total bilirubin (mg%)	3.5	0.5	0.8	2.3	0.8	0.6
Serum iron (μg%)	80	—	—	—	—	—
Sickling	+	+	—	+	—	+
Fetal haemoglobin ( )	8.4	0	0	.5	1.5	0
Haemoglobin A_2 ( )	6.5[a]	5.5[a]	4.5[a]	4.7[b]	6.7[b]	
Haemoglobin S ( )	79.2	28.5	0	67.3	0	
Haemoglobin A ( )	5.9	68	93.5	8	9[b]	
G-6-PD screening test	normal	normal				

Not performed.
Performed by the method of starch gel electrophoresis (14)
Performed by the method of DEAE-cellulose chromatography (16)

Case 2

G. S., 5 year old Turkish girl was brought to the Outpatient Department of Çapa Internal Clinic of Istanbul Medical School because of anaemia and pains in bone and joints. She is the sister of patient with sickle cell- beta thalassaemia disease who was reported elsewhere (17). Physical examination revealed well developed and well nourished white girl with mildly subicteric sclerae. The spleen was enlarged two finger breadths below the costal margin. The laboratory findings are summarized in Table I.

Family study. The father had sickle cell trait and the mother was beta-thalassaemic heterozygote. The parents' haematologic findings are summarized in Table I.

Discussion

The genetic pattern and the results of haemoglobin analyses of our two patients are consistent with the diagnosis of sickle cell-beta thalassaemia disease. Haemoglobin analysis revealed that a high

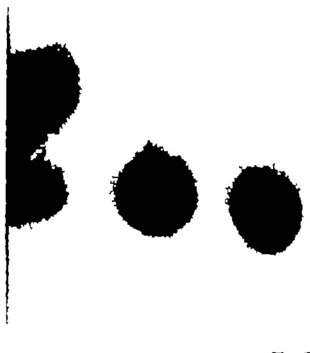


Fig. 2. Starch gel electrophoresis (borate buffer 0.025 M, pH 8.6. From left to right: A+S+A₂ (Sickle cell trait: Hb A 39.5 %, Hb S 57 % and Hb A₂ 3.5 %); A+F+S+ increased A₂ (sickle cell–beta thalassaemia disease, normocytic type: case 1: Hb A 5.9*, Hb F 8.4 %, Hb S 79.2 % and Hb A₂ 6.5 %); F+S+A₂ (sickle cell-anaemia: Hb F 6.5 %, Hb S 89.7 % and Hb A₂ 3.8*). The gel was photographed without staining.

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RBC (10^6/mm³)	3.40	4.70	4.90	3.40	4.10	4.60
Hb (g%)	8.9	13.5	12	7.5	11.3	13.6
WBC/mm³	9 200	8.000	7.000	10.800	7.200	6.500
Reticulocytes (%)	6.5	1	1.5	3.6	3	0.5
Haematocrit ( )	31.5	42	44	26	33	43
MCV (μ^3)	92.6	87	89	75	80	93
MCHb. ($\mu\mu$g)	26.2	27	24	22	27	29
NRC/100 WBC	4	0	0	1	0	0
Polychromatophilia	+	—	—	+	—	—
Poikilocytosis	—	—	—	+	—	—
Hypochromia	—	—	+	++	++	—
Microcytosis	—	—	—	++	++	—
Basophilic stippling	—	—	—	+	+	—
Target-cells	++++	—	+++	+++	++	—
Sickle-cells	+	—	—	—	—	—
Osmotic fragility (% NaCl)	0.4–0.16	0.4–0.3	0.4–0.2	0.4–0.18	0.44–0.22	0.4–0.3
Total bilirubin (mg%)	3.5	0.5	0.8	2.3	0.8	0.6
Serum iron (μg%)	80	—	—	—	—	—
Sickling	+	+	—	+		+
Fetal haemoglobin ( )	8.4	0	0	25	1.3	0
Haemoglobin A_2 ( )	6.5[2]	3.5[2]	4.5[2]	4.7[3]	6.7[3]	
Haemoglobin S ( )	79.2	28.5	0	62.3	0	
Haemoglobin A ( )	5.9	68	95.5	8	92	
G-6-PD screening test	normal	normal				

Not performed.
Performed by the method of starch gel electrophoresis (14)
Performed by be method of DEAE-cellulose chromatography (16).

Case 2

G. S 5 year old Turkish girl was brought to the Outpatient Department of Çapa Internal Clinic of Istanbul Medical School because of anaemia and pains in bone and joints. She is the sister of patient with sickle cell- beta thalassaemia disease who was reported elsewhere (17). Physical examination revealed well developed and well nourished white girl with mildly subicteric sclerae. The spleen was enlarged two finger breadths below the costal margin. The laboratory findings are summarized in Table I.

Family study. The father had sickle cell trait and the mother was beta-thalassaemic heterozygot The parent haematologic findings are summarized in Table I

Discussion

The genetic pattern and the results of haemoglobin analyses of our two patients are consistent with the diagnosis of s ckle cell-beta thalassaemia disease Haemoglobin analysis revealed that a high

haemoglobin S fraction (62.3 and 79.2%), moderately elevated haemoglobin F (8.4 and 25%), increased haemoglobin A_2 content (4.7 and 6.5%) and a low haemoglobin A fraction (5.9 and 8%). The genetic pattern of these two patients showed that one of the parents had sickle cell trait and the other exhibited the haematologic findings of heterozygous beta thalassaemia including increased haemoglobin A_2. On the other hand, the clinical manifestations of these two patients with sickle cell beta thalassaemia disease were identical and resembling mostly those of sickle cell anaemia, such as pains in bones and joints, abdominal pain and fever. In addition to these, one of them (case 1) exhibited cardiac manifestations and findings which resembled closely those found in mitral stenosis.

Despite the similarity of clinical features, genetic patterns and the results of haemoglobin analyses in our two patients with sickle cell beta thalassaemia disease, the type of anaemia was entirely different. The type of anaemia was normocytic in the first case whereas it was hypochromic microcytic in the second. In other words, the anaemia of case 1 resembled that found in sickle cell anaemia whereas the anaemia of case 2 was similar to that seen in intermediate type of COOLEY's anaemia. Here arises the following question: How can the occurrence of two different types of anaemia, one normocytic and the other microcytic, be explained in sickle-cell-beta thalassaemia disease? The following possibilities may be considered in explaining the above mentioned differences concerning the type of anaemia in some cases of sickle cell beta thalassaemia disease.

(a) The presence of more than one gene responsible for beta thalassaemia. A comparison of the values of mean corpuscular volume obtained in beta thalassaemic parents of these two patients with sickle cell beta thalassaemia disease revealed a different picture. In accordance with the values of their offspring (the patients), the beta thalassaemic parent of case 1 showed a normal value of mean corpuscular volume (89 μ^3) whereas a decreased mean corpuscular volume of 80 μ^3 was found in the beta thalassaemic mother of case 2. In other words, despite an evident microcytosis in the beta thalassaemic parent of case 2, this haematologic criterion was not found in the beta thalassaemic parent of case 1. From this standpoint there was a complete similarity between the patients and their beta thalassaemic parents.

Although microcytosis is considered a unique feature of heterozygous form of thalassaemia gene by some investigators (18,19) there are numerous beta thalassaemic individuals without showing this haematologic criterion (20 21) Therefore, we may presume that there are at least two kinds of genes responsible for beta thalassaemia one presenting microcytosis in heterozygous state and the other without showing this haematologic criterion.

On the other hand, the above emphasized similarity between the values of mean corpuscular volume obtained in the patients with sickle cell beta thalassaemia disease and their beta-thalassaemic parents was not found in every family with this haemoglobinopathy studied (17)

Despite the discrepancy between the values of mean corpuscular volume obtained in some patients with sickle cell- beta thalassaemia disease and their beta thalassaemic parents, we believe that the occurrence of different types of anaemia may be explained by the presence of several genes responsible for beta-thalassaemia.

(b) The presence of environmental and genetic modifiers This hypothesis has been advanced in explaining some difficult *genetic problems in thalassaemic patients (7 15 20) But in our* present state of knowledge it is impossible to give an exact definition of them.

Summary

Two patients with sickle cell- beta thalassaemia disease are reported. Despite the similarity of clinical pictures, genetic patterns and the results of haemoglobin analyses in these patients, the type of anaemia was entirely different it was normocytic in one case whereas it was hypochromic microcytic in the other The possible genetic explanations for the occurrence of different types of anaemia in sickle cell-beta thalassaemia disease are discussed.

Zusammenfassung

Es wird über zwei *Patienten mit Sichelzell-β-Thalassämie* berichtet. Trotz der Ähnlichkeit von klinischem Bild, Erbmodus und den Ergebnissen der Hämoglobinanalyse war die Art der Anämie bei diesen zwei Patienten vollkommen verschieden sie war normozytär beim einen Fall und hypochrom-mikrozytär beim anderen Fall Die möglichen genetischen Erklärungen für das Vorkommen verschiedener Anämieformen bei der Sichelzell-β-Thalassämie werden diskutiert.

Résumé

Rapport sur deux malades atteints de bêta-thalassémie falciforme Malgré la similitude du tableau clinique de l'hérédité et des résultats des analyses de l'hémoglobine chez ces malades, le type d'anémie était complètement différent Les possibilités d'une explication génétique de l'existence de différentes formes d'anémie dans la bêta-thalassémie falciforme sont discutées.

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Identification of Ferritin within Gaucher Cells

An Electron Microscopic and Immunofluorescent Study

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Diffuse Prussian blue staining of the characteristic cells in 5 cases of Gaucher's disease has been reported by one of us (1). This citation of three additional cases reaffirms that finding and, by the use of a fluorescent antibody technic and electron microscopy, identifies the reactive substance as ferritin. In some electron micrographic studies of this disorder dense particles similar to those presently noted have been demonstrated. However, their presence was not emphasized (2, 3). In others, none were visible in the published figures (4), but often the magnification was too low (5, 6) to visualize them had they been ferritin micelles. The presence of iron within the Gaucher cells is compatible with recent reports that the cerebroside characteristic of this disorder may result from the accumulation of erythrocyte glycolipid in reticuloendothelial cells (7–9), suggesting a common derivation of both iron and lipid from red blood cells.

Case Reports

Case 1. 40 year old female, splenectomized at age 17 for thrombocytopenia. At that time, the diagnosis of Gaucher's disease was made. Postoperatively, her platelet level increased. Since then, she has been asymptomatic except for the development eight years ago of pain in her right hip and knee accompanied by radiographic evidence of bone resorption. There is a more recent history of a mild anemia treated with an oral iron preparation with benefit. Other than for the presence of a moderate number of Gaucher cells, no marked hematologic abnormality was present in the bone marrow aspirate obtained for this study. Her hemogram was: hematocrit 41.5, reticulocytes 1.3, WBC 11 050/mm³ and platelets 204 000/mm³.

Case 2. 46 year old man who developed easy bruisability. Thrombocytopenia and splenomegaly were discovered and Gaucher cells noted on a bone marrow aspirate ob-

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tained shortly before admission to the Georgetown University Hospital. His spleen, surgically removed, provided tissue for this study. His hemogram was: hematocrit 45.5 %, reticulocytes 1.7%, WBC 4,700/mm³ and platelets 15,000/mm³. Postoperatively the thrombocytopenia disappeared.

Case 3. 54 year old woman in whom Gaucher's disease was diagnosed 30 years previously. She developed severe pancytopenia: hemoglobin 8.2, WBC 2,700/mm³ and platelets 88,000/mm³. Her spleen, surgically removed elsewhere several years ago, had been maintained in the frozen state since then. During the year prior to splenectomy she received iron medication for the anemia without improvement.

Methods

General staining reactions. Mallory adaptation of the Prussian blue procedure (10), Wright stain of smears, hematoxylin and eosin and PAS staining of sections were performed.

Indirect fluorescent antibody technic. An alum precipitate was prepared from cadmium-free recrystallized ferritin (Pentex, Inc., Kankakee, Ill.). This was diluted with saline to concentration of 0.15 mg N/ml and used for immunizing rabbit four times weekly for four weeks (11). One week after the last injection, the rabbit was fasted and bled. The serum was dialyzed overnight at 4 °C against large volume of isotonic saline. One ml of antiserum contained 52.4 μg of antibody nitrogen. Serum was also obtained from an unimmunized rabbit. Globulin fractions from both were prepared by ammonium sulfate fractionation and then absorbed with acetone-dried rat liver powder to minimize nonspecific fluorescence (12). Methanol-fixed smears or frozen sections were exposed to non-fluorescing rabbit anti-ferritin globulin followed by fluorescent goat anti-rabbit serum (12) (Microbiological Associates, Inc., Bethesda, Md.) diluted 1:8 with 2.5% bovine serum albumin in buffered saline for optimal fluorescence. The controls were: (1) non-fluorescing normal rabbit globulin followed by the fluorescent goat anti-rabbit serum, and (2) the latter alone. These were essentially negative.

The tissues were examined with an American Optical microscope with Nerni-Clarke mercury vapor light source, AO 774 (Schott OG1) barrier and AO 702 (Schott BG-12) exciter filters.

Electron microscopy. Portions of the freshly-obtained bone marrow from Case 1 and spleen from Case 2 were fixed in cold buffered osmium tetroxide and embedded in Epon. Sections were cut on a Porter Blum ultramicrotome using glass knife. They were mounted on grids and examined with Philips 75 electron microscope or RCA EMU 2B electron microscope.

Photography. Electron micrographs were taken on Kodak medium-contrast lantern slides developed in D-11. Ordinary light microphotographs were made with Kodachrome X or KR 135 film. Polaroid 3000 film was used for the fluorescent microphotographs.

Results

In all three cases, most Gaucher cells were diffusely stained by Prussian blue (Fig. 1). Neither the hematoxylin and eosin nor PAS procedures performed on adjacent sections demonstrated observable differences between cells that did or did not stain with Prussian blue. Judging by the intensity of the staining, the quantity of iron as well as of PAS-positive material, varied among the characteristic cells (Fig. 1) independent of any observable local cause such as



Fig 1 Case 2. Spleen section. Variations in staining suggest the presence of different quantities of iron (ferritin) among the Gaucher cells. Prussian blue (× 120)

relationship to blood vessels. Hemosiderin and lipofuscin granules were rare in Cases 1 and 2 who were not anemic, but common in Case 3 whose cells were most markedly stained by Prussian blue.

Prussian blue staining of pieces of spleen from Cases 2 and 3 resulted in gross staining throughout, but particularly in the red pulp indicating concentration of ferric iron in that location.

All the cases had many Gaucher cells with varying degrees of cytoplasmic fluorescence (Fig 2 3) No autofluorescence was noted. The immunologic technic demonstrated iron in fewer cells than did the histochemical method

Electron micrographs of Cases 1 and 2 confirmed the presence of ferritin in the cytoplasm of most Gaucher cells. The electron dense particles were more often concentrated in the periphery particularly in and near the numerous pseudopods that at times form the external surface of the cell In both, larger aggregates of ferritin were infrequent (Fig 4) In the vicinity of sequestered erythrocytes (Fig 5) were numerous dense areas which may contain ferritin and perhaps, lipid. Individual ferritin micelles were usually not conspicuously associated with organelles such as the endoplasmic reticulum or mitochondria (Fig 4) Some characteristic cells had no significant ferritin, indicating that lipid and iron accumulation do not necessarily parallel each other Gaucher



Fig 2 Case 1 Bone marrow smear Fluorescence of Gaucher cell (400)

Fig 3 Case 3 Spleen smear The intracellular hemosiderin granules I) are more prominent than the diffuse ferritin F Fluorescence 1500

cells contain large membrane limited zones with elongated tubules resembling end plasmic reticulum. Such fascicles may be respon sible for the characteristic striations f the cells under light microscopy



Fig. 4. Case 2. Numerous ferritin micelles (arrows) are present in the cytoplasm, particularly in the periphery. Occasional ferritin aggregates (FA) are present. There are zones (Z) containing tubular structures resembling endoplasmic reticulum. The latter are of larger diameter than the internal cristae of mitochondria (M). The zones are demarcated by a single membrane (m) in contrast to the double mitochondrial membrane (15000)

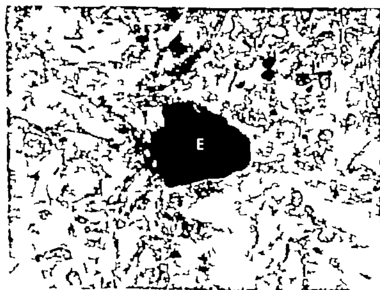


Fig. 5. Case 1. An erythrocyte (E) is sequestered between several Gaucher cells. There are numerous radiodense particles, some of which appear to be lysosomes. There are ferritin aggregates (FA) and perhaps lipid, presumably originating from the adjacent erythrocyte (6000

Discussion

The tendency to a peripheral localization suggests that much of the ferritin may enter Gaucher cells by ropheocytosis (13) from adjacent cells, plasma or tissue fluid. Another potential source could be erythrophagocytosis (1, 14). However, most of the observed Gaucher cells show no evidence of this process, perhaps reflecting rapid digestion of red cell fragments as in reticuloendothelial cells in which rupture of red cells into two or more parts occurs in 5–15 min (15). The presence of iron in Gaucher cells, as well as of acid phosphatase (16) and nonspecific esterase (17), and the occurrence of a positive PAS reaction (18) may reflect their relationship to macrophages (19, 20) and other reticuloendothelial cells (21). Phagocytosis (21) and proliferation (22, 23) favor those histochemical reactions in the latter and presumably likewise in the abnormal cells derived from that system (24).

The negative Prussian blue reaction in *infants* with Gaucher's disease (18, 25) and in children with Niemann-Pick's disease (26, 27) indicates that the occurrence of intracellular iron is not basic to such reticuloendothelial disorders, but that chronicity may be necessary for its deposition. Likewise, the lack of anemia and the 1.3 and 1.7 ⁄ reticulocyte levels in Cases 1 and 2 suggest that normal or slightly increased iron turnover occurring over years or decades may by itself cause significant accumulation of ferritin in the characteristic cells. Case 2 had no history of iron medication, so such therapy played no role in his cellular deposits.

The spleen of Case 3 had appreciable intracellular and extracellular hemosiderin as well as intracellular ferritin, so that a hemolytic basis to her anemia is likely. However, she was also the oldest of the cases and had received iron medication, thereby possibly augmenting the accumulation of iron which was observed. The marked deposition in her spleen resembles that seen in hemosiderosis and in hemochromatosis, in which there may be a maximum concentration of free ferritin in the cytoplasm of affected cells with addition of further ferritin resulting in the formation of aggregates (28, 29). Ferritin and apoferritin, being immunochemically indistinguishable (30), are included in the specific precipitate (31) in the fluorescent antibody procedure. Both also occur in hemosiderin (32, 33), perhaps explaining the fluorescence of the intracellular aggregates of Case 3 (Fig. 3).

In Gaucher's disease, the basic defect of lipid metabolism is said to be a deficiency of an enzyme (7, 8) converting ceramide-glucose derived from red blood cells to ceramide. If erythrocyte glycolipid were the precursor of the cerebroside (9) in the Gaucher cell, then its iron may likewise largely derive from ingested red cells. However, in some characteristic cells iron cannot be demonstrated. Therefore either not all the lipid in this disorder derives from ingested erythrocytes or removal of ferritin from the cells occurs more rapidly than does removal of the lipid.

Finally, the present observation that more iron is visualized with the Prussian blue reaction than with a fluorescent procedure is similar to that made by CRATO in a study of erythroblastosis fetalis (34). This may be because much of the stainable iron may be non-protein bound or because the antiserum presently employed has greater specificity or lesser sensitivity. It appears to be a poorer indicator than is Prussian blue which can reveal as little as 0.1 pg of iron (35).

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Summary

In three adults with Gaucher's disease, two of them having no anemia, ferritin has been identified within many of the characteristic cells. Its accumulation is not a basic defect because of its absence in some otherwise typical cells. The increased cellular ferritin may derive from chronic ingestion of erythrocytes or of plasma or interstitial fluid iron. Both intracellular and extracellular iron may accumulate when increased hemolysis or long duration of the disease exist, the liberated iron being deposited in the tissues in the storage forms of ferritin and hemosiderin.

Zusammenfassung

Bei drei Erwachsenen mit Gaucherscher Krankheit, von denen zwei keine Anämie hatten, konnte in den charakteristischen Zellen Ferritin nachgewiesen werden. Seine Speicherung stellt keine grundlegende Störung dar, da es in einigen der typischen Zellen fehlte. Der erhöhte Gehalt von Ferritin in den Zellen kann bedingt sein durch chronische Phagozytose von Erythrozyten, durch Aufnahme von Plasma oder interstitieller Flüssigkeit. Intra- und extrazelluläres Eisen kann sich ansammeln bei gesteigerter Hämolyse oder langer Dauer der Krankheit, wobei das freigesetzte Eisen in den Geweben als Ferritin oder Hämosiderin abgelagert wird.

Résumé

Chez 3 adultes tteints de la maladie de G ucher dont deux valent pas d anémie de la ferritine pu être démontrée dans beaucoup des cellules caractéristiques. Etant bsente dans certaines cell les d utre part typique son accumulation ne représente pas une perturbation essentielle Le contenu augmenté de ces cellules en ferritine peut être causé par une phagocytose hronique d érythrocytes, par l' bsorption de fer plasmatique ou de fer enant d fluide interstitiel. Le fer in ra ou extracellulaire peut accumuler lors d'une hémolyse accrue ou d'une maladie de longue durée le fer libéré éta t déposé dans les tissus sous forme de ferritine ou d'hémosidérine

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Characterization of Glucose 6-Phosphate Dehydrogenase in Sardinian Children with Congenital Nonspherocytic Haemolytic Anaemia

F. Schettini and T. Meloni

Congenital nonspherocytic haemolytic anaemia (CNHA) in Caucasian subjects may be associated with a deficiency of erythrocyte glucose-6-phosphate dehydrogenase (G-6-PD) (1–14). The first studies carried out on the characterization of the erythrocyte G-6-PD of a boy with CNHA have shown several qualitative characteristics different from primaquine sensitive Negroes and normal persons (7, 15). Subsequent investigations have shown in subjects with CNHA and G-6-PD deficiency qualitative characteristics of the G-6-PD that differ from the enzyme of the subjects of the racial group (16, 17). Such enzymes may be defined as variants (17). G-6-PD exhibits many functional peculiarities in G-6-PD deficient Sephardic, Jewish and Sardinian subjects and in some enzyme deficient Greeks and is designated as a *Mediterranean Variant* (18). In these racial group and especially in Sardinian subjects it is possible to observe congenital nonspherocytic haemolytic anaemia associated with a deficit of G-6-PD (6, 9, 13).

It is the purpose of this paper to compare the chemical characteristics of the G-6-PD of two Sardinian children with CNHA and erythrocytic G-6-PD deficiency with those of the G-6-PD Mediterranean variant. Both parents also have been studied.

Material and Methods

CNHA subjects. We has studied children, boy and girl, and their parents. Major clinical features of these subjects has been referred to earlier by P zos and Zacchello (9). The subjects re of Sardinian origin.

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Collection of samples. Blood from each subject was injected aseptically into plastic bag containing ACD solution (acid-citrate-dextrose solution, Formula A USP 0.15 ml per ml of blood). The bag was placed in ice and shipped by air express to Dr H. N. KIRKMAN (Oklahoma City Okla., USA). The samples were examined within 5 days of venipuncture.

Red cells were isolated, hemolyzed with 9 volumes of solution consisting of 10 μM NADP and 7 mM β-mercaptoethanol, and cleared of stroma, as described by KIRKMAN *et al.* (18)

Enzymic assay and purification. Activity of G-6-PD was measured from the rate of generation of NADPH, as followed 340 μm in Beckman DU spectrophotometer A unit of activity is defined as the amount of enzyme necessary for the reduction of one micromole of NADP per min (7)

G-6-PD was isolated from hemolysates by adsorption on diethylamino-ethyl cellulose, elution and subsequent ammonium sulphate fractionation (19).

All the preparations of G-6-PD were dialyzed before each experiment. The dialysis solution consisted of 0 05 M TRIS (hydrochloride) pH 8.0 0.10 μM NADP 7 mM β-mercaptoethanol and 2.7 mM EDTA (N salt, pH 7.0)

Electrophoretic studies of G-6-PD The electrophoresis of the enzyme was carried out using the method of KIRKMAN and HENDRICKSON (20) and used in previous work (21).

Kinetic and lability studies. The Michaelis constant for G-6-P (Km G-6-P) was obtained by observing enzymic rates at each of six different concentrations of G-6-P (0 0285 to 1.83 mM) The pH optima were determined in duplicate at different pH values in cuvettes containing mixture of TRIS, phosphate and glycine in final concentrations of 0.05 M each. The solutions were adjusted to the desired pH 7.5. The Km value for G-6-P was estimated from plots of the reciprocal of the rate against reciprocal of substrate concentration (22)

Substrate analog utilization. The rate of utilization of 2-deoxy glucose-6-phosphate (2d-G-6-P) was estimated by determination of rates in assay mixtures containing 0.7 mM G-6-P or 2d-G-6-P This rate was expressed as percentage of the rate of utilization of G-6-P

Results and Discussion

The clinical features of the CNHA subjects and of their parents are listed on Table I.

The G-6-PD activities were 7.2 for the male and 74 6 for the female affected by CNHA. The father and the mother showed respectively 131 and 117 units per 100 ml of red cells Table II The yield and the specific activity of the G-6-PD of CNHA are summarized in Table II With the small amount of blood of low G-6-PD activity it was not possible to do a full characterization of the enzyme The following parameters were studied.

pH Optima Activity of each dialyzed enzyme was determined in duplicate at pH values in the range 5 5 to 10 0 The pH optimum curve of the CNHA male exhibited a bimodal curve similar to Mediterranean subjects Table II and Fig 1 The pH optimum curves of the CNHA female and of the parents were normal Table II

Table I
Clinical features of CNHA subjects and their parents.

Personal history	CNHA male	CNHA female	F ther	Mother
Age t time of stud (years)	3	8	39	.5
Age when jaundice or anemia w first noted (months)	1	25	–	
Neonatal jaundice	no	no		
Episodes of anaemia	yes	yes	no	no
Splenomegaly	yes	yes	no	no
Haemoglobin (g/100 ml)	7.60	8.40	14 4	13..

Table II
Comparativ characteristics of G-6-PD

	CNHA male	CNHA female	F ther	Mother
Units of G-6-PD 100 ml of red blood cells	7..	74 [illegible]	131	117
Yield of G-6-PD	18	1	1	14
Specific activity of partially purified G-6 PD units mg protein	0 0135	0 0[illegible]63	0.31	0.1[illegible]
Purification fold	8[illegible]	47	103	58

Adul con rol alues for samples collected and shipped in similar way can be obtained from preceden article 18

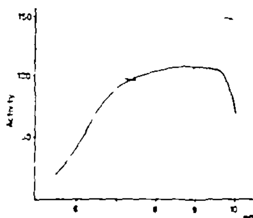


Fig 1 pH optimum curves f CNHA male broken line and for CNHA female [illegible] line Activ is expressed as percentage of the activity pH 5

Michaelis constant for G-6-P The enzymic rates of the CNHA subjects and of their parents at various concentrations of G-6-P are shown in Table IV The CNHA male had Km s values for G-6-P lower than normal. Values for the CNHA female and for the pa rents did not differ from the values of normal Caucasians (18)

Substrate analog utilization The rate of utilization of 2d-G-6-P of the CNHA male was very elevated while CNHA female and the parents showed normal values (Table V)

Electrophoretic migration of G-6-PD The migration on vertical starch-gel electrophoresis of the enzyme was of type B in the CNHA male and of type B in the CNHA female and in the parents (Fig 2)

In two children with CNHA we have observed a low activity of G-6-PD in the male and an intermediate activity of the enzyme in the female

Table III
Effects of pH on rates of CNHA and control G-6-PD

Subjects	Average rate t pH 7.5 (OD × 10³/min)	Relative rate per cent of the rate t pH 7.5									
		pH 5 5	pH 6.0	pH 6.5	pH 7.0	pH 8.0	pH 8.5	pH 9.0	pH 9.5	pH 9.75	pH 10.0
CNHA male	0.60	49	93	105	100	101	104	117	151	157	138
CNHA female	3.68	13	33	73	90	106	115	117	118	95	66
Father	2.55	6	35	72	90	102	104	117	102	82	44
Mother	2.26	11	31	69	89	111	116	116	102	78	44
Avg 29 normal Caucasian men		10.4	35.3	71 4	93.0	105 5	110.7	113 6	99.0	74.8	44.6
Standard deviation ±		2.2	4.4	5 0	3.3	2.5	4.5	6.0	5.9	7.9	8.5

Table IV
Effect of G-6-P concentration on rates of CNHA and control enzyme

Subjects	G-6-P concentration (mM) (rates, OD 10³/min)						Km
	1.88	0.188	0 113	0 0565	0.0377	0.0285	(µM)
CNHA male	2 57	2.60	2.28	2 08	1.99	1.89	12
CNHA female	1 76	1.36	1 18	0.874	0 76	0 62	55
Father	3.50	2.40	2.35	1.65	1 28	1 08	62
Mother	1 42	1 18	1.01	0 74	0 62	0 31	56
Normal							56–76
Mediterranean variant							19–26

Table V
Substrate analog utilization.

S bjects	A erage rate with G-6-P ( )	(OD × 10⁴/min) with 2d-G-6-P (b)	Relati utilization of 2d-G-6-P (b/a × 100)
CNHA male	7.33	1.92	26.2
CNHA female	33.0	1 11	3.2
F ther	36.9	0.85	2.3
Mother	21.5	0.46	2.2
Normal			<4
Mediterranean variant			23–37



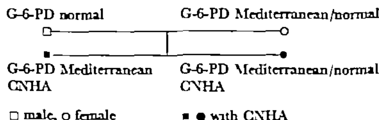
Fig. Patterns of erythrocyte G-6-PD migration on ertical starch gel electrophoresis Channels contain the following samples.
1 CNHA male *2* father of CNHA, *3* Negro type A, *4* Negro type A, *5* normal subject type B, 6 CNHA mother *7* normal subject type B, *8* CNHA male type B The types 1 2 and 6 are B

The G-6-PD of the male shows a bimodal curve of pH optimum a low Km for G-6-P and a marked relative utilization of the 2d-G-6-P These data strongly indicate that it is one of the usual *Mediterranean variants* In the girl and in the parents the character

ristics of the G-6-PD are similar to normal Sardinian subjects. The girl with CNHA has level of G-6-PD and qualitative characteristics of the enzyme compatible with the heterozygous state. The mother has a good level of G-6-PD and normal qualitative characteristics. The father is normal both for the level of G-6-PD and for the qualitative characteristics.

The peculiar qualitative characteristics of Mediterranean G-6-PD in heterozygous female are masked by relative large amounts of normal G-6-PD.

The inheritance of G-6-PD may be proposed in this family as sex linked and the genetic transmission of CNHA as an autosomal recessive gene.



Qualitative characteristics of the G-6-PD have been observed in some subjects with CNHA and enzymatic variants have been exhibited. The *Oklahoma variant* shows an elevated Km's value for NADP G-6-P and 2d-G-6-P and unusual pH optimum curve and moderate thermolability (17). The *Chicago I variant* is characterized by very rapid loss of activity during storage of heating. A *thermolabile CNHA variant* has been reported (23) with a slightly abnormal pH optimum curve and with a moderate elevation of Km for G-6-P. An *Essen variant* has been described by Boyer *et al.* (24) with very lability and electrophoretic migration slightly faster than normal.

The G-6-PD in our observations does not show different characteristics from Mediterranean variant that is the prevalent in the Mediterranean populations and particulary in Sardinian subjects (18).

We wonder if the G-6-PD deficiency of the male CNHA is strictly related to congenital nonspherocytic haemolytic anaemia or is merely coincident. A support to this doubt is given by the recent observation of two Negroe subjects with CNHA and apparently coincident (Type A-) G-6-PD deficiency (25). It is not possible to

Table I
Substrate analog utilization.

Subjects	Average rate with G-6-P (a)	(OD x 10⁴/min) with 2d-G-6-P (b)	Relative utilization of 2d-G-6-P (b/a x 100)
CNHA male	7.33	1.92	26.2
CNHA female	35.0	1.11	3.2
Father	36.9	0.83	2.3
Mother	21.5	0.40	2.[illegible]
Normal			<4
Mediterranean variant			23-37



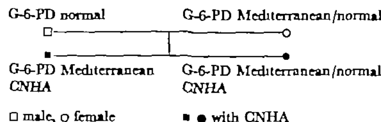
Fig. Patterns of erythrocyte G-6-PD migration on vertical starch gel electrophoresis. Channels contain the following samples.
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The G-6-PD of the male shows a bimodal curve of pH optimum, a low Km for G-6-P and a marked relative utilization of the 2d G-6-P. These data strongly indicate that it is one of the usual *Mediterranean variants.* In the girl and in the parents the character

istics of the G-6-PD are similar to normal Sardinian subjects. The girl with CNHA has level of G-6-PD and qualitative characteristics of the enzyme compatible with the heterozygous state. The mother has a good level of G-6-PD and normal qualitative characteristics. The father is normal both for the level of G-6-PD and for the qualitative characteristics.

The peculiar qualitative characteristics of Mediterranean G-6-PD in heterozygous female are masked by relative large amounts of normal G-6-PD

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resolve this doubt or the define exactly the significance of the G-6-PD deficit in the pathogenesis of the congenital nonspherocytic haemolytic anaemia

Acknowledgement. We are grateful to Dr. HENRY N. KIRKMAN, Department of Pediatrics, University of North Carolina, Chapel Hill, U.S.A., for collaboration to provide these data and for helpful suggestions, and to Dr. F. P[illegible]ZON and F. ZACCHELLO for providing the patients.

Summary

The biochemical characteristics of the G-6-PD has been evaluated in two Sardinian children affected by congenital nonspherocytic haemolytic anaemia and G-6-PD erythrocyte dificiency and in their parents. The G-6-PD of the male is one of the usual 'Mediterranean variants'. In the girl and in the parents the characteristics of the enzyme are similar to normal Sardinian subjects.

Zusammenfassung

Bei zwei sardinischen Kindern mit kongenitaler nicht sphärocytärer hämolytischer Anämie und G-6-PD-Mangel der Erythrocyten sowie bei deren Eltern wurden die biochemischen Eigenschaften der G-6-PD untersucht. Bei dem Knaben stellt die G-6-PD eine der üblichen «mediterranen Varianten» dar. Bei dem Mädchen und bei den Eltern entsprechen die Eigenschaften des Enzymes denjenigen gesunder Sardinier.

Résumé

Les propriétés biochimiques de la G-6-PD ont été étudiées chez deux enfants sardes souffrant d'anémie congénitale hémolytique non-sphérocytaire et d'un manque en G-6-PD érythrocytaire ainsi que chez leurs parents. La G-6-PD de l'enfant mâle est une des «variantes méditerranéennes» courantes. Chez la fille et les parents, les propriétés du ferment correspondent à celles de l'enzyme de sardes normaux.

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Übergang einer arzneimittelempfindlichen Erythropathie bei Glukose-6-Phosphatdehydrogenase-Mangel in eine chronische hämolytische Anämie

D. Busch unter Mitarbeit von Karin Bohe

Eine *pathologische Verminderung* der *Glukose-6-Phosphatdehydrogenase-Aktivität* der roten Blutzellen ist eine der häufigsten erblichen Stoffwechselabweichungen. Nachdem durch die entscheidenden Arbeiten der Arbeitsgruppe von Beutler, Alving, Dern u. a. der corpusculäre Charakter dieses Erythrozytendefektes erkannt und das Unvermögen der roten Blutzellen, unter besonderen Bedingungen, u. a. bei Einwirkung bestimmter Medikamente Glutathion in der reduzierten Form zu erhalten, als ein wesentliches Stoffwechselmerkmal dieser Zellen aufgedeckt worden war, gelang es 1956 Carson *et al.* als Ursache der beobachteten Stoffwechselabweichungen und der Anfälligkeit der Erythrozyten einen Mangel an dem Enzym Glukose-6-Phosphatdehydrogenase in den betroffenen Zellen nachzuweisen.

So häufig dieser Defekt in der Weltbevölkerung auftritt (nach Carsons Zusammenstellung 1960 in über 100 Millionen Menschen), so sehr ist sein Vorkommen an bestimmte Rassen gebunden, und er wird gerade in Deutschland und allgemein in Nordeuropa nur außerordentlich selten angetroffen. In Deutschland sind uns nur 16 publizierte Fälle bekannt (Gehrmann *et al.* 1965, Schievelbein *et al.* 1963, 1965, Hennemann *et al.* 1964a, b, Schröter, 1965, Walter *et al.* 1966).

Unterschiedliche Varianten des Enzymdefektes treten klinisch unter verschiedenartigen Bildern auf. In der großen Mehrzahl der Fälle kommt die Hyperhämolyse erst durch Hinzutreten äußerer Noxen in Gang, sie ist zeitlich eng begrenzt und nach Wegfall der Noxe (Medikamente, Vegetabilien u. a.) ist die Erythrozyten

lebenszeit nur unwesentlich – klinisch völlig asymptomatisch – verkürzt. Vergleichsweise extrem selten besteht hingegen das klinische Bild einer hereditären nichtsphärozytären hämolytischen Anämie. Nur etwa 60 derartige Fälle sind in der Weltliteratur bisher bekannt*. In Deutschland gehören vier der erwähnten Fälle in diese Gruppe.

Wir beobachteten bei einer jetzt 82 Jahre alten Frau deutscher Abstammung eine in mehrfacher Hinsicht bemerkenswerte Form des Defektes. Erstmals im hohen Alter von 78 Jahren war aus voller klinischer Gesundheit heraus eine arzneimittelinduzierte hämolytische Krise aufgetreten, die sich dann mehrfach in typischer Weise wiederholte. Im weiteren Verlauf jedoch ging die Symptomatik in die einer chronischen, noxenunabhängigen hyperregeneratorischen Anämie mit acholurischem Ikterus und Retikulozytose über, die interessante Fragen, auch hinsichtlich ihrer Klassifizierung, aufwirft.

Methoden

Enzymaktivitäten der Erythrozyten. Aufarbeitung des Blutes und Erythrolyse wie früher beschrieben (Busch, 1964). Leuko- und Thrombozytenabtrennung jedoch an der Baumwollsäule, wie andernorts angegeben (Busch und Pelz, 1966). Der Leukozytenanteil lag dann unter 1 pro 4×10^4 Erythrozyten. Die verwendeten Enzymteste sind im einzelnen andernorts angegeben (Busch, 1966a).

Enzymaktivitäten der Leukozyten. Leukozytenanreicherung nach Löhr *et al.* (1964). Enzymteste wie oben.

Metabolitbestimmungen. Perchlorsäure Extraktion des Blutes nach Hohorst *et al.* (1959). Beschreibung der Teste für die Metabolitbestimmungen im einzelnen bei Busch (l. c.).

Bestimmung der *Glykolyserate* (Laktatbildung der Erythrozyten aus Glukose bei 37° C, pH 7,35) wie früher angegeben (Busch, 1964). *Glutathionstabilitätstest* und Heinz-Körper-Test nach Beutler (1957 resp. 1955).

Substanzen

Die Substanzen hatten den Reinheitsgrad p. a. und wurden überwiegend, ebenso die Hilfsenzyme, von der Firma Boehringer Mannheim, bezogen, NEM von der Firma Fluka, Bern, 5,5-DTNB von der Firma Serva, Heidelberg.

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* Vgl. Übersicht Busch, 1966c.

Abkürzungen: *APH* Acetylphenylhydrazin, *ADP* Adenosindiphosphat, *ATP* Adenosintriphosphat, *5,5-DTNB* 5,5-Dithio-Nitrobenzoesäure, *GSH* reduziertes, *GSSG* oxydiertes Glutathion, *F-6-P Ald.* Fruktose-6-Phosphat-Aldolase, *GAPDH* Glycerinaldehydphosphatdehydrogenase, *G-6-PDH* Glukose-6-Phosphatdehydrogenase, *GR* Glutathionreduktase, *HK* Hexokinase, *NEM* N-äthylmaleinsäureimid, *6-PGDH* 6-Phosphoglukonatdehydrogenase, *PGK* Phosphoglyceratkinase, *PHK* Phosphohexokinase, *PK* Pyruvatkinase, *UMI* Urobilin[illegible].

Kasuistik

P. L. M. Wö..., geb. 1883, Frau, Anamnese: Die Patientin war bis zum Jahre 1960 gesund und hatte körperlicher Arbeit gewachsen. Über eine Blutkrankung, insbesondere über hämolytische Phasen in früherer Zeit, ist nichts bekannt. Im 78. Lebensjahr (1960) wurde eine Herzbehandlung mit einem ... haltigen Kombinationspräparat (Eucard) eingeleitet und weiterhin regelmäßig ... verordnet. In dieser Zeit trat bei zunehmender Müdigkeit eine Anämie auf. Unter Behandlung einer Bronchopneumonie mit Par... entwickelte sich innerhalb weniger Tage eine schwere Gelbsucht. Deren Zusammenhang mit der Par... wurde ... erkannt, die Diagnose Bronchopneumonie revidiert und ursächlich eine Cholangitis angenommen, obwohl sonst keinerlei Symptome vorlagen, die diese Diagnose unterstützen konnten. Drei weitere Jahre waren bis auf eine konstant geringe ikterische Hautfarbe erscheinungsfrei. 1963 traten plötzlich Fieber und Somnolenz auf. Wegen Apoplexieverdachtes applizierte der Hausarzt u. a. die Vitamine C und K parenteral. Nach 12 Stunden ... schlagartig ein schwerer Ikterus auf. Trotz der jetzt festgestellten Retikulozytose wurde dessen hämolytischer Charakter wiederum ... erkannt. Die Symptomatik klang rasch ab, wurde jedoch durch eine erneute Par...behandlung re... ... Später haben Gaben von 100 mg Furadantin täglich (wegen einer Pyelonephritis) angeblich keinen Ikterus provoziert. Eine geringe ikterische Verfärbung der Haut habe aber, wie erwähnt, seit 1961 gleichmäßig bestanden ... vermutlich unterhielt das fortlaufend applizierte ...haltige Herzpräparat den hyperhämolytischen Prozeß. Als erneut eine Pneumonie hinzutrat, wurde Furadantin durch Par... ersetzt und damit nun zum dritten Mal ein sch... ikterischer Schub ausgelöst. Jetzt wurde bei der Patientin, die seither in unserer Beobachtung steht, eine Verminderung der Glukose-6-Phosphatdehydrogenase der Erythrozyten festgestellt. ... ist, daß der hyperhämolytische Prozeß nun ... der ... sämtlicher Medikamente, die hierbei für die Auslösung einer Hämolyse in Betracht kommen, nicht mehr zum Stillstand kam.

Klinischer Befund[1]: 83jährige, noch rüstige Patientin. Erhebliche Kyphose. Blässe, geringer Sklerenikterus. Absolute Arrhythmie des Herzens, weitere Befunde altersentsprechend unauffällig. Leber und Milz nicht vergrößert.

Hämatologische Befunde (Tabelle 1): Hb 9–12 g%, Retikulozyten 40–220‰, seit etwa 1 Jahr auch ohne Einwirkung äußerer Noxen um 100‰. MCH 32 pg, MCHC 30 g/100 ml Erythrozyten, MCV 10? µm³, MCD 7,9 µm (Price-Jones-Kurve Doppelgipfel bei 7 und 8 µm), MCT 2,8 µm, sphärischer Index 0,36. Leukozyten 7500, Thrombozyten 460000/µl. Osmotische Resistenz 0,52–0,32% NaCl (Kontrolle 0,48–0,34), mechanische Resistenz 8,5 ... 3,8). Direkter Antiglobulintest mit verschiedenen Coombsseren häufig wiederholt stets negativ. Kälteagglutinintiter unter 8 (Prof. Dr. Sch...). Autohämolyse (dens.) ohne Zusatz 8,9%, mit Glukose 1,1 ..., mit ATP 0,5 ... (entspricht Typ I nach Selwyn und Dacie). Säureresistenz normal. Hämoglobinelektrophorese (Doz. Dr. ..., Tübingen): HbA₂ 2,3 ..., HbF ..., kein anomales Hämoglobin. Blutgruppe: 0 Rh ... Knochenmark (Doz. Dr. W...): extrem gesteigerte normoblastäre Erythropoese, keine qualitativen, insbesondere keine megaloblastischen Veränderungen. Im Serum: direktes Bilirubin negativ, indirektes Bilirubin 1–4 mg%, in den letzten Monaten konstant um 2 mg%. Haptoglobin vermindert (10 mg% ...). Serum-Fe anfangs vermindert (verringerte Eisenresorption), nach Therapie ... ohne Einfluß auf die Anämie ... normalisiert. Weitere blutchemische

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[1] Ich danke den behandelnden Stationsärzten Doz. Dr. Dot... und W..., sowie Dr. C... und Dr. D... für die Unterstützung bei den klinischen Untersuchungen.

Tabelle I

Hämatologische Daten und biochemische Befunde an den Erythrocyten der Patientin M. Wö. Seit 19. 10. 1963 keine Medikament Applikation, welche Hämolysen auslösen kann.

		11. 11. 1964	8. 10. 1965	5. 11. 1965	17. 1. 1966	22. 3. 1966
Hb (g%)		11,2	11,9	9,5	11,5	10,9
Ery (10^6/µl)		3,3	3,6	2,9	3,9	3,[illegible]
Reti (‰)		142	145	105	119	118
Bilir (mg%)		1,6	2,3	2,0	1,9	2,0
Enzyme in Erythr.	Normalbereich ($\bar{x} \pm 2s$)					
G-6-PDH	87—207	58	89	66	42	32
6-PGDH	87—175	204	266	220	233	212
GR (NADPH)	74—142	197	222		249	
GR (NADH)	34—66	65	74		99	
HK (37 °C)	11—31	114	96		23	33
PHK	137—293			143	373	
FDP-Ald.	42—166			165	275	
GAPDH	2600—4600			1870	4970	3180
PGK	1930—3100			3280	3230	
Enolase	90—166			83	124	
PK	195—335			437	647	
Glykolyserate	21—47			61		
Metabolite i. Erythr.						
ATP	1030—1550	1370		1140		1290
ADP	162—396	166				
AMP	10—92	50				
ATP/ADP	2,7—7,1	8,3				
GSSG	9—61	23	31			
GSH	1630—2910	1680	1500		1310	1620
% GSH n. 2h APH	68—107	48	57	45	63	56

Enzyme in IE/10^{10} Ery (25 °C, HK 37 °C, pH 7,5) *Glykolyserate* in nMol Laktat/ml Ery × min (37°C, pH 7,35) *Metabolite* in nMol/ml Ery Glutathionstabilitätstest n. Beutler (1957)

Untersuchungen unauffällig, [illegible]. keine Hinweise auf Cholostase. Galle Rö. o. B. Im dunklen Urin vermehrt Urobilinogen, jedoch kein Bilirubin nachweisbar. Urobilinkörperausscheidung im Stuhl auf die 8fache mittlere Norm erhöht (UMI ~ 500)

Stoffwechseluntersuchungen. Die Aktivität der Glukose-6-Phosphatdehydrogenase der roten Blutzellen ist – zu verschiedenen Zeiten – auf 30–60% der mittleren Aktivität für Normalpersonen vermindert (Tabelle I). Dieser Befund ist jedoch wegen der hier vorliegenden Retikulozytose und der hierbei normalerweise stets erhöhten Glukose-6-Phosphatdehydrogenase-Aktivität irrelevant. Der Defekt besteht auch in den Leukozyten. Die Restaktivität beträgt hier 10% der mittleren Norm (Tabelle II). Sämtliche weiteren, für Erythrocyten angegebenen Enzyme wurden auch in Leukozyten gemessen und waren hier ebenfalls normal.

Chinidin (26 µg/ml) führte wie APH zum pathologischen GSH Abfall (63%)

Tabelle II

Leukocyten-Enzymaktivitäten bei Patientin M. W. im Vergleich zu Normalpersonen.

Enzym (Leukocyten-Extrakt)	Normalwerte N	Mittel	Bereich	NP*	Pat. M. Wo. ♀ (17.1.66)
G-6-PDH	16	15	10—23	13	1,5
6-PGDH	16	7	6—11	8	10
GR (NADPH)	3	10	7—14	14	30
GR (NADH)					3,5
HK (37°C)				1,4	1,4
PHK				7	13
FDP-Ald.				21	18
GAPDH	3	80	[illegible]—103	103	173
PGK	3	83	[illegible]	[illegible]	[illegible]
Enolase	3	8	7—11	11	18
PK	16	51	30—115	26	14

Enzymaktivität in IE/10⁹ Leuk. (25°C, HK 37°C, pH 7,5)

Bereich = Extreme der Einzelwerte resp. deren Mittel an N Normalpersonen (NP)

* Simultan mit Pat. M. Wo. an einer NP gemessene Werte

Tabelle III

Wärmelabilität der Glukose-6-Phosphatdehydrogenase (G-6-PDH) in Erythrocyten-Rohhämolysaten bei hereditärer nichtsphärocytärer hämolytischer Anämie mit G-6-PDH-Mangel (Pat. J. H.) und der Pat. M. Wo. im Vergleich zu Normalpersonen (NP).

	G-6-PDH-Aktivität nach 40°C-Inkubation 0 min	10 min	20 min	40 min
J. H., ♂	5 (100%)	2 (40%)	1,5 (30%)	1 (20%)
M. Wo.,	16 (100%)	16 (100%)	14 (88%)	10 (63%)
NP	33 (100%)	30 (91%)	[illegible] (85%)	23 (70%)
NP	38 (100%)	36 (95%)	30 (79%)	26 (70%)

Enzymaktivitäten in Arbeitseinheiten und % der Werte vor Inkubation.

Der Gehalt der Erythrocyten an reduziertem Glutathion ist deutlich erniedrigt, die Glutathionstabilität (unter Einwirkung von Acetylphenylhydrazin auf intakte Erythrocyten) stark herabgesetzt, die Innenkörperbildung gleichzeitig pathologisch vermehrt (5 Innenkörper in > 40% der Zellen). Der Gehalt an oxydiertem Glutathion (GSSG) war in den nativen Erythrocyten normal. Die Glykolyserate ist entsprechend der erheblichen Verjüngung der gesamten Zellpopulation erhöht, der Gehalt an Adeninnukleotiden und der ATP/ADP-Quotient normal (Tabelle I).

Michaeliskonstanten. Die Bestimmung der Michaeliskonstanten der Glukose-6-Phosphatdehydrogenase der Erythrocyten ergab Normalwerte (K_M [NADP] = 6,5 × 10^{-4} M, K_M [G-6-P] = 4,1 × 10^{-4} M; normal K_M [NADP] = 3,4—7,1 × 10^{-4} M, K_M [G-6-P] = 3,5—5,6 × 10^{-4} M (Marks, 1961, in Übereinstimmung mit den eigenen Ergebnissen). Die Michaeliskonstanten wurden an Rohhämolysaten gemessen.

Tabelle IV
G-6-PDH Aktivität in ungereinigten Erythrozyten-Hämolysaten in Abhängigkeit vom pH.

pH		5,6	6,0	6,5	6,9	7,5	7,9	8,5	8,9	9,4
Aktivität	Norm (3 NP)	13	24	68	90	*100*	103	109	119	107
(%)	Pat. M. Wö.	10	24	67	100	*100*	95	91	86	86

Aktivitätsangabe in % der Werte bei pH 7,5, 25 °C, TRIS-PO_4-Glycin-Puffer

Wärmelabilität. Auch die Wärmelabilität des Erythrozytenenzyms war bei der Patientin M. Wö. normal (Tabelle III). Im Gegensatz dazu fanden wir diese bei einem Patienten mit hereditärer nichtsphärozytärer hämolytischer Anämie bei Glukose-6-Phosphatdehydrogenasemangel abnorm gesteigert (J. H., ♂, Patient von Dr. Kornöcz, II. Med. Univ.-Klinik Wien, Direktor Prof. Dr. Fellinger; das Blut wurde uns freundlicherweise zur Untersuchung zur Verfügung gestellt).

pH-Aktivitätskurve. Die pH-Kurve der G-6-PDH-Aktivität zeigt bei der Patientin M. Wö. eine Differenz zur Norm (Tabelle IV). Untersuchungen am gereinigten Enzym und Isoenzymtrennungen sind hier jedoch erforderlich: die normale breite pH-Kurve scheint eine Superposition verschiedener pH-Optima zu bilden (Maximum bei pH ~ 9, Schulter bei pH ~ 7,5), die Patientenkurve könnte durch asymmetrische Verminderung eines Aktivitätsanteiles ohne echte Verschiebung des Optimums bedingt sein.

Familienuntersuchungen. Die Messung der Glukose-6-Phosphatdehydrogenase bei den Blutsverwandten unserer Patientin (einem Sohn, einer Tochter und deren Tochter) ergab Normalwerte. Hiermit wird weiterhin die Heterozygotie der Patientin bezüglich des G-6-PDH-Defektes belegt, da von homozygot defekten Frauen keine erbgesunden Söhne abstammen können. Anamnestisch gibt es auch bei anderen Familienangehörigen keine Hinweise auf hämolytische Krankheitsbilder.

Diskussion

Hämolytische Erkrankungen auf dem Boden verminderter Glukose-6-Phosphatdehydrogenase-Aktivität stellen sich klinisch in unterschiedlicher Symptomatologie dar. In weit überwiegender Anzahl sind die Betroffenen in der Regel völlig asymptomatisch – Anämie und Ikterus treten krisenhaft und temporär auf nach Verabfolgung bestimmter Arzneimittel («arzneimittelempfindliche Erythropathie») bzw. nach Genuß ungekochter Feld- (= Fava) bohnen («Favismus») kommt es nach unterschiedlicher Latenzzeit, die von Art und Menge der einwirkenden Noxe abhängt, zum Erythrozytenzerfall. Diese Hämolyse «aus heiterem Himmel» kommt binnen kurzem wieder zum Stehen, da die aus der Regeneration nachfließenden roten Zellen – nach allgemeiner Annahme auf Grund ihrer höheren G-6-PDH-Aktivität – zunächst gegenüber der schädigenden Noxe resistent sind. Die Erythrozytenlebenszeit ist ohne einwirkende zusätzliche Schädigungen nur

geringfügig verkürzt. Vergleichsweise extrem selten sind bei Glukose-6-Phosphatdehydrogenasemangel hereditäre nichtsphärozytäre hämolytische Anämien. Hier besteht schon spontan eine konstante Hyperhämolyse mit Anämie und Ikterus. Sie wird durch die genannten Noxen, oft auch durch unspezifische Einwirkungen wie Infekte, nur zusätzlich aktiviert. Vermutlich handelt es sich bei beiden Bildern um prinzipiell differente Varianten des Defektes (Kirkman *et al.* 1960, Marks 1964).

In unserem Fall wird ein scheinbarer Übergang beider klinischer Formen beobachtet. Die Patientin war bis zum 77. Lebensjahr symptomfrei. Zwar fehlen frühere objektive Befunde, aber nichts weist auf einen chronischen hyperhämolytischen Prozeß bis zu dieser Zeit hin. Die dann zunächst unter dem Bild der »arzneimittelempfindlichen Erythropathie« auftretende Symptomatik, wahrscheinlich vier Jahre lang gleichmäßig unterhalten durch Chinidin, krisenhaft verschlimmert durch Vitamin K (?) und v. a. durch Chloramphenicol, wurde später von der Einwirkung äußerer Noxen unabhängig und ging nun in das Symptomenbild der chronischen nichtsphärozytären hämolytischen Anämie über.

Ob es sich hier um eine echte »primäre« hereditäre nichtsphärozytäre hämolytische Anämie bei Glukose-6-Phosphatdehydrogenasemangel oder um eine Spielart der arzneimittelempfindlichen Erythropathie handelt, steht zur Diskussion. Folgendes weist auf das letztere hin:

a) Die Restaktivität der Glukose-6-Phosphatdehydrogenase ist hier relativ hoch. In den bekannten Fällen von hereditären nichtsphärozytären hämolytischen Anämien fehlt die G-6-PDH-Aktivität zumeist völlig, zumindest weitgehend, und zwar – typischerweise – bereits in den Retikulozyten, so daß auch eine verjüngte Population keinen Aktivitätsanstieg zeigt.

b) Die Anämie tritt hier bei einer Frau auf. Die bisher bekannten hereditären nichtsphärozytären hämolytischen Anämien mit G-6-PDH-Mangel wurden bei Männern beobachtet. Die Androtropie des Defektes erklärt sich aus seinem X-chromosomalen Erbgang.

c) Michaeliskonstanten und Wärmelabilität des Enzyms sind normal. Eine nicht wesentliche Abweichung der pH-Aktivitätskurve (s. o.) kann vor eingehenderen Untersuchungen am gereinigten Enzym und Isozymauftrennungen nicht als Ausdruck eines pathologischen Enzymproteins gewertet werden. – Hinsichtlich der Klä-

rung der Frage nach den Ursachen der so unterschiedlichen Phänomenologie des Glukose-6-Phosphatdehydrogenasemangels waren Befunde der Arbeitsgruppen von MARKS und von KIRKMAN wegweisend (Lit. s. o.) diesen Autoren war es gelungen, erstmals am Menschen pathologische Enzymvarianten nachzuweisen. In Fällen von hereditären nichtsphärozytären hämolytischen Anämien wurden pathologische Isoenzyme der Glukose-6-Phosphatdehydrogenase erkannt, die in dieser Form bei den arzneimittelempfindlichen Erythropathien mit G-6-PDH Mangel nicht nachweisbar waren. Nach Art und Ausprägung unterschiedliche pathologische Abweichungen der Substrataffinitäten zu Glukose-6-Phosphat und (oder) NADP der Thermolabilität, der pH Aktivitätskurven sowie des elektrophoretischen Verhaltens ließen eine größere Anzahl verschiedener Varianten des Defektes abgrenzen (vgl. Übers. MARKS, 1964) Die bei dem oben genannten, von uns untersuchten Patienten der Wiener Klinik (hereditäre nichtsphärozytäre hämolytische Anämie mit G-6-PDH Mangel, G-6-PDH Aktivität der Erythrozyten 14% der mittleren Norm) bestehende pathologische Wärmelabilität des Enzyms ordnet sich in die bekannten Daten ein und weist auch hier analog auf ein anomales Isoenzym hin. Die Michaeliskonstanten für G-6-P und NADP waren dabei normal. Unsere Patientin M Wö dagegen ließ in den bisherigen Untersuchungen, abgesehen von der besprochenen, noch nicht sicher einzuordnenden Abweichung des pH-Optimums, keine pathologischen Abweichungen der bei hereditären nicht sphärozytären hämolytischen Anämien nach bisheriger Erfahrung am häufigsten veränderten Enzymeigenschaften erkennen. Selbstverständlich ist die Prüfung weiterer Qualitäten erforderlich

Die bemerkenswert späte Erstmanifestation der Erkrankung veranlaßte uns zu vergleichenden Untersuchungen an anderen über 80 Jahre alten Frauen Zwischen den mittleren Aktivitäten der Glukose-6-Phosphatdehydrogenase der Erythrozyten dieser und jüngerer Normalpersonen findet sich kein Unterschied. Das gleiche gilt für Glutathiongehalt und stabilität. Wir haben somit keinen Zweifel, daß es sich hier nicht um ein erworbenes Phänomen handelt, obwohl keine Belege aus der Familienuntersuchung beigebracht werden können, die – wie erwähnt – Normalwerte ergaben. Sie konnten sich jedoch nur noch auf einen Sohn und eine Tochter erstrecken

Die Frage, auf welche Weise die ausgeprägte hyperhämolytische Anämie ohne die Einwirkung äußerer Noxen bei der bestehen

den, im Vergleich zur typischen hämolytischen Glukose-6-Pho phatdehydrogenase-Mangel-Anämie relativ hohen Restaktivit des Enzyms der roten Zellen unterhalten wird, bleibt zunäch offen. Es muß wohl angenommen werden, daß der Defekt zuvor 7 Jahre lang symptomfrei bestanden hat, wie es auch bei zahllose anderen heterozygoten Frauen mit quantitativ vergleichbarer De fektausprägung die Regel ist. Aber hier kommt es nicht zur hämo lytischen Krise und eine chronische hämolytische, von zusätzliche Einwirkungen unabhängige Anämie ist u. W. dabei nicht beob achtet worden (vgl. LARIZZA, 1961). – Über biochemische Unter suchungen zum Hämolysemechanismus der defekten Erythrozyte wird getrennt berichtet.

Meiner Assistentin, Frau KARIN BOER, danke ich für ihre zuverlässige und fleiß Mitarbeit. Die Untersuchungen wurden finanziell von der Deutschen Forschung gemeinschaft unterstützt.

Zusammenfassung

Eine 83jährige Patientin zeigt klinisch den Übergang einer typischen arzneimitte empfindlichen Erythropathie bei Glukose-6-Phosphatdehydrogenasemangel mit pa sageren, durch Medikamente ausgelösten hämolytischen Krisen in eine chronisch noxenunabhängige hyperregeneratorische Anämie. Eine Arzneimittelhämolyse man festierte sich erstmals im 78. Lebensjahr. 6 Jahre später beherrscht bei strenger Verme dung hämolysierender Noxen ein konstanter hämolytischer Ikterus das klinische Bil Die Glukose-6-Phosphatdehydrogenaseaktivität der Erythrozyten beträgt bei ausg prägter Retikulozytose um 30% die der Leukozyten um 10% der Norm. Die Unte suchung eines Sohnes und einer Tochter ergibt normale G-6-PDH-Aktivitäten de Blutzellen. Bei der Patientin sind Michaeliskonstanten und Wärmelabilität der Res aktivität des Enzyms normal. Die pH-Kurve ist gering verändert. Unterschiede gegen über bekannten hereditären nichtsphärocytären hämolytischen Anämien mit Glukose-6 Phosphatdehydrogenasemangel werden hervorgehoben.

Summary

A woman of 83 showed the clinical manifestations of a transition from a typica drug-sensitive erythropathy with glucose-6-phosphate dehydrogenase deficiency an brief drug-induced haemolytic crises to a chronic hyperregenerative anaemia indepen dent of noxious influences. Haemolysis caused by a drug first occurred when she was 78 and six years later, despite rigorous avoidance of all haemolytic agents, haemolytic jaundice was constantly present. Glucose-6-phosphate dehydrogenase activity of the erythrocytes, with a marked reticulocytosis, was about 30% and that of the leukocyte about 10% of normal. A son and daughter who were also examined both had normal blood-cell G-6-PDH activity. The Michaelis constants and heat lability of the residual

enzyme activity were normal in this patient. The differences compared with the known G-6-PDH deficient hereditary non-spherocytic haemolytic anaemias are emphasized.

Résumé

Chez une malade de 83 ans atteinte d'une érythropathie médicamenteuse due à un manque de glucose-6-phosphate-deshydrogénase (G-6-PD) et qui subit plusieurs crises hémolytiques passagères par des médicaments, se développa une anémie hyper régénératrice sans cause apparente. Une hémolyse médicamenteuse se manifesta pour la première fois à l'âge de 78 ans. 6 ans plus tard un ictère hémolytique constant domine le tableau clinique quoique tout facteur capable de provoquer une hémolyse soit strictement évité. L'activité de la G-6-PD des érythrocytes est de 30% de la valeur normale quand la réticulocytose est prononcée, celle des leucocytes de 10% de la valeur normale. L'activité de la G-6-PD déterminée chez un fils et une fille de la malade était normale. Chez la malade, les constantes de Michaelis et la labilité à la chaleur de l'activité résiduelle du ferment étaient normales. Des différences par rapport à l'anémie hémolytique héréditaire non-sphérocytaire avec manque de G-6-PD sont indiquées.

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Christmas Disease in a Girl with Female Karyotype

C. Rozman, R. Castillo, M. Ribas-Mundó and J. Surós

Classically, hemophilia A, resulting from antihemophilic globulin deficiency (factor VIII), and hemophilia B or Christmas disease (from PTC or factor IX deficiency) are determined genetically by a recessive gene linked to the X chromosome, and therefore, exceptionally seen in females.

Not long ago, Ulutin *et al.* (1) reviewed the world literature and found only 16 cases of female hemophilia A, reporting another one. To theirs we must add a case recently described by Whisell *et al.* (2). In our bibliographic research we have found only 3 cases of female hemophilia B or Christmas disease (3, 4, 5). We think, therefore, it might be of interest to present a female karyotype patient with sporadic hemophilia B.

Case Report

Ma. Dolores J. B., a seven-year-old girl. No bleeding tendency was found in either her father' or mother' family. A 14-year-old brother and a 12-year-old sister had no hemorrhagic symptoms.

She was the product of a full term pregnancy with difficult delivery. In her fourth day of life she developed laryngeal spasms with cyanotic crisis which recurred two days later. Before she was one-year-old, her parents noted a marked tendency to bruise and develop hematomata. At one year of age, she injured one finger which bled for 15 min. At 2 years, she had severe bleeding for 3 days following an injury at the frenulum of the upper lip. In her 4th year, severe bleeding from an injury at the same site required three blood transfusions. Thereafter, she suffered extensive and multiple hematomata and repeated bouts of hemarthrosis of the left ankle. At the age of 6, she bled severely after a tooth extraction, and three more blood transfusions were needed.

She was first seen by one of us (C. R.) on April 23rd 1964. On physical examination, we found painful swelling at the right ankle and left knee, mild atrophy of the right calf and obvious limping. The phenotype was feminine.

Laboratory Investigations

The following data were obtained elsewhere when the patient was one year old whole blood coagulation time 27 min, bleeding time 3½ min, platelets 360,000/mm³

Our laboratory investigations (24-IV 1964) gave the following results Capillary fragility normal, bleeding time (Ivy) 5 min. Clot retraction normal. Platelets 350,000/mm³ Whole blood coagulation time 38 min. Prothrombine (Quick) 100% Prothrombine consumption time 18 (normal over 25). Fibrinogen 4 32 g %. Fibrinolysis normal (Von Kaulla = 3 h). Thromboelastogram: r = 8.5 mm; k = 2.5 mm m = 70 mm. The bleeding time tested on several occasions was always normal. The in vivo platelet adhesiveness by the Borchgrevink method (6) was 41 % (normal 20—50%).

Thromboplastin generation tests. The TGT by the Biggs and Douglas method (7) showed pathological results clearly indicating deficiency in the patient serum (Fig. 1) The factor IX level (8) was 2 The TGT was not corrected *in vitro* by adding to the patient serum hemophilic B serum but brought to the normal range with addition of hemophilic A serum (Fig. 2) The infusion to the patient of hemophilic B plasma did not alter the Biggs and Douglas test, but it was partially normalized by the infusion of hemophilic A plasma. Her father and mother serum TGT was normal, with 100% activity (Fig 3)

Genetic studies. Buccal smears from the patient revealed female pattern of nuclear sex-chromatin. Chromosome analyses were conducted on peripheral blood leukocyte culture according to the method of Moorhead *et al.* (9). Counts of 55 metaphase figures revealed modal number of 46 chromosomes. A microscopical analysis of these cells revealed that only 4 chromosomes were present in the G group. An apparently normal female karyotype was confirmed by photography in 8 cells (Fig. 4)

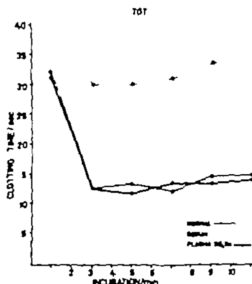


Fig. 1 Deficient thromboplastin generation with the patient serum

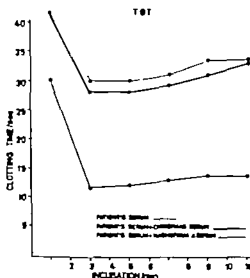


Fig. 2. The thromboplastin generation deficiency is not corrected by adding Christmas serum, but the test becomes normal by adding hemophilia A serum.

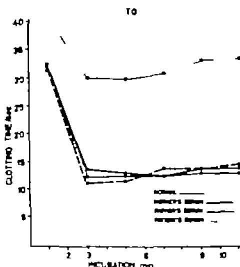


Fig. 3. The thromboplastin generation test with parent serum is normal.

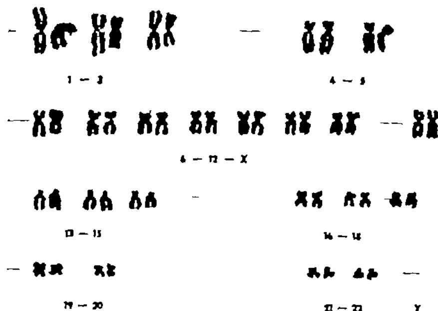


Fig 4 Karyotype analysis of the patient showing female chromosome pattern (46 xx) cultured from peripheral blood leukocytes.

Discussion

When we consider a possible case of female hemophilia, we must first rule out an error of sex diagnosis. NILSSON *et al* (10) described a female hemophilia A with female phenotype, but the analysis of the karyotype revealed a male chromosome pattern this case was therefore considered as a testicular feminization. In the case we are presenting there is a female sex-chromatin pattern and a female karyotype which exclude this possible error

The likelihood of an angiohemophilia (prolonged bleeding time and deficiency of factor VIII or IX) should also be considered *In our patient, the bleeding time was repeatedly normal and the* platelet adhesiveness in vitro was also in the normal range (in angiohemophilia it is consistently abnormal) The possibility of a circulating anticoagulant can also be excluded by the results of TGT (Fig 2)

It is apparent from the clinical and laboratory data that our patient meets the requirements for the diagnosis of hemophilia B or Christmas disease. Hemophilia in females can be genetically class-

fied in three types (1) homozygous female hemophilia, (2) heterozygous female hemophilia, (3) sporadic female hemophilia.

These three possibilities have been described for the hemophilia A. An occasional homozygous female hemophilia could possibly be the offspring of an affected male and a carrier female. Earlier these cases were considered always lethal, although some of them have been reported. The first case was described by MERSKEY (11) when he restudied a family previously investigated thereafter other authors have substantiated this possibility Heterozygous female hemophilia is found in female carriers of the hemophilic trait, i. e daughters of hemophilic father or carrier mother Usually but not always, since the female carriers have no bleeding tendency they can be detected only by a low AGH (factor VIII) level. However in 2 % of these cases, the factor VIII level is lower than 25% (12) and there are exceptional cases repoted with levels of 6 % (13) 4% (2) and even 1.5—2 % (14) with bleeding tendency Sporadic female hemophilia has been explained by the assumption of a mutation of one or probably both hemophilia loci on the X chromosomes of the propositae When the mutation occurs in one or both loci, the result is a sporadic heterozygous female hemophilia or a sporadic homozygous female hemophilia, respectively Sporadic hemophilia A in females has been first reported by QUICK and HUSSEY (15) since then other cases have been published including the recent presentation of another normal female karyotype patient, by ULUTIN *et al.* (1)

The three cases of hemophilia B in females reported earlier include two genetically determined and a sporadic one. The first paper (3) refers to a 22-year-old girl with very mild bleeding tendecy that followed only teeth extractions the first blood transfusion was required at adult age. Her factor IX level was 9%. Her father suffered from Christmas disease, and, therefore, the daughter was *probably a female carrier (heterozygous) with very mild* symptoms. The second case (4) was a 6-year-old girl with severe hemophilia and repeated hemarthrous, requiring in total 10 blood transfusions. Her factor IX level was 2—3 %. Her mother and sister were hemophilic B carriers and her father was not affected. This case can be considered as a heterozygous female hemophilia B with severe clinical manifestations and a very low factor IX level or according to the author's suggestion, as a homozygous female hemophilia B due to mutation of the paternal hemophilia locus asso-

ciated with the maternal hemophilia trait. The third case (5) of hemophilia B in females was a 10-year-old girl with traumatic hematomata and severe hemorrhages after minor surgery. No familial history of bleeding tendency was detected. The sex-chromatin body in buccal smears was positive. This case should therefore be considered as the first one published of sporadic female hemophilia B. Unfortunately the negative familial history was not proven by laboratory investigations, and, therefore, the possibility of a heterozygous female hemophilia B cannot be ruled out.

The patient we are referring to can also be regarded as a sporadic female hemophilia B case. There is no family history of bleeding tendency and the factor IX level of the propositae's father and mother is normal. It is difficult to ascertain whether one or both hemophilic loci are affected in this case. The severe bleeding tendency and the very low factor IX level favours the assumption of a homozygous state but the possibility of a double mutation is very low. We considerer more likely the mutation in only one locus, with an increased expressivity of the hemophilic trait. The decrease of factors VIII or IX level and the bleeding tendency in heterozygous female hemophilia have been recently explained by the LYON's theory (16). She suggested that one of the two X chromosomes in each cell of the female subject is inactivated at random early in embryonic development. In the heterozygous female hemophilia the X chromosome, with either the mutant or the normal gene, might be inactivated. The factor VIII or IX level is possibly related to the proportion of cells with active or mutant genes in the tissues responsible for the production of these clotting factors. The inactivation af almost all normal X chromosomes might be responsible for the very low factor VIII or IX levels in the heterozygous female hemophilia.

Summary

The authors refer to 7-year-old girl exhibiting severe hemorrhagic symptoms and very low factor IX level (hemophilia B or Christmas disease) and no familial history of bleeding tendency. The possibility of sporadic female hemophilia B is suggested.

Zusammenfassung

Es wird über ein 7 Jahre altes Mädchen berichtet, das schwere hämorrhagische Symptome und einen sehr niedrigen Gehalt an Faktor IX aufwies (Hämophilie B oder Christmas-Krankheit), wobei in der Familie keine Blutungsneigung vorkam. Es wird die Möglichkeit einer sporadischen weiblichen Hämophilie B erwogen.

Résumé

Les auteurs rapportent le cas d'une enfant de sept ans atteinte d graves symptomes hémorragiques et ayant un taux très bas en facteur IX (hémophilie B), une tendance ux hémorragies existant pas dans la famille. La possibilité d'une hémophilie B sporadique chez une personne de sexe féminin est prise en considération.

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XVth Colloquium, Bruges
Mai 3–7 1967

Protides of the Biological Fluids
General Topics

(A) The proteins of membrane and surface structures: (1) Morphology and composition of membranes. – (2) Membrane function. Round table: The proteins of membrane and surface structures.

(B) The proteins of the complement activity. (1) Chemistry of complement components. (2) The biological functions of complement. Round table: The nomenclature of complement components.

(C) Techniques. (1) Preparative techniques. (2) New methods and techniques. (3) Clinical chemical methods. Round table: (a) Preparative techniques. (b) Clinical chemical methods.

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Institute of Pathology, University of Cologne
(Acting Director: Prof. Dr. med. G. RUDOLPH)

PAS Positive Erythroblasts in Kidney Diseases

H. O. KLEIN and A. HELLER

The pathogenesis of normochromic anemia often present in acute and chronic glomerulonephritis or chronic sclerosing glomerulonephritis or in chronic pyelonephritis, interstitial nephritis, and in nephrosis or in cases of acute kidney failure has not yet been clarified (8, 24). The degree of severity of anemia is related to the extent of renal parenchyma loss, but it does not definitely depend on the severity of uremia (8). With as little as an average creatinine retention of 1.5 mg % the volume of circulating erythrocytes drops by one fourth and with 12 mg % creatinine retention as much as half the norm (9). Cytologic examinations of bone marrow from patients suffering from kidney diseases have shown varying results: normoplastic, hypoplastic and aplastic red marrow has been found (9, 10, 11, 13, 23, 31). The level of erythropoietin was found to be considerably reduced in the plasma of patients suffering from kidney disorders (16, 18, 23, 27, 29). Additionally, it has been established that the life-span of erythrocytes is considerably shorter in these patients (6, 7, 9, 12, 22, 24).

To our knowledge cytochemical examinations of erythroblasts in the bone marrow of patients with kidney diseases have not yet been made. For that reason this paper will report on such examinations of patients who suffered from diseases of the kidney or who died as a result of such diseases. In doing so the cytochemical results will be related to the degree of severity of damage to the kidney parenchyma. Patients who suffered or died from hematological or other organic diseases that did not affect the kidneys served as control groups.

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PAS Positive Erythroblasts in Kidney Diseases

H O Klein and A. Heller

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To our knowledge cytochemical examinations of erythroblasts in the bone marrow of patients with kidney diseases have not yet been made. For that reason this paper will report on such examinations of patients who suffered from diseases of the kidney or who died as a result of such diseases. In doing so the cytochemical results will be related to the degree of severity of damage to the kidney parenchyma. Patients who suffered or died from hematological or other organic diseases that did not affect the kidneys served as control groups.

Material and Methods

Numerous sternal bone marrow smears were prep red from clinical patients and the dead. From the latter the bone marrow could be emoved only bout 18 hours after death. The following stains were used

(1) PAS-reaction (M MA) according to PEARSE method (28)
Fixation air-dried smears in 100° methanol 5 min,
Stain placed in perjodic acid solution (0.8) 10 min,
washed in 70° alcohol,
placed in HOTCHKISS reducing solution 1 min,
washed in 70° alcohol,
placed in SCHIFF' sol tion 15 min,
washed in running w ter 20 min,
nucleus stained in M hemalaun 8 min,
washed in running water 6 min.
(2) PAS-F -stain according to MERKER s method (26)
(3) PAS-reaction after saliv treatment (1 h at 37 C)
(4) PAS-reaction after lipid extraction (in methanol-chloroform 4 h t 60 °C)
(5) PAS-reaction after combined saliv treatment and lipid extraction.
(6) PAS-reaction after acetylation and deacetylation (28) to prov presence of 1 2 glycols.
(7) BEST' carmine stain (32) to prove presence of glycogen.
(8) Sudan black B stain accordi g to the SHEEHAN nd STOREY method (33) to prov the presence of lipids.
(9) Toluidine blue stain (28) to prov presence of acidic mucopolysaccharides.
(10) F LGEN s stain (32) to prov the presence of DNA.
(11) MILLON reaction (28) to prov the presence of tyrosine
(1) Ninhydrin-SCHIFF-reaction (28) to establish the presence of free NH_2-groups
(13) PAPPE HEIM stain (32)

I evaluating the slide smears stained according to these different methods total of least 500 erythroblasts t varying stages of maturity were counted at time and the erythroblasts found to be positive stained were given in percentages. In order to judge correctly differences in the intensity of staining of the erythroblasts of different cases using different staining methods, smears from several cases (topsy cases and clinical patients) were always stained in one staining procedure. The strong positi ity of the neutrophil granulocytes served as control for the regularly executed PAS stain. Erythroblast staining of differing intensity as nevertheless observed in PAS staining and was evaluated according to QUAGLINO and H YHOE (30) scoring system and expressed in an index

0 group erythroblasts with negativ staining reaction.
1st group erythroblasts with pale red staining of cytoplasm or 1 3 red granules in the cytoplasm.
2nd group erythroblasts with medium red staining of the cytoplasm or 3–6 red granules.
3rd group erythroblasts with strong red staining of he cytoplasm similar to tha of the neu rophil granulocytes or 6–10 red granules.

The guidelines for calculating the index are as follows The number of cells obtained by counting t least 100 erythroblasts for the four groups mentioned above are to be multiplied by the group number The sum of the products of the individual groups is the Index The possible index values vary between 0 and 300. The significance of the index is that gradual differences in the intensity of staining of the cytoplasm can be expressed numerically

The enzymatic removal of glycogen with saliva was carried out on other smear preparations. After one hour treatment t 37 °C all glycogen was removed as the neutro-

phil granulocytes showed that were not stained in the following PAS stain. The neutrophil granulocytes accordingly contain glycogen exclusively as also shown by HECKNER (20).

The extraction of the lipids at 60 °C in methanol-chloroform mixture was fully complete after 24 h. The sudan black B stain carried out afterwards did not stain any more lipids on the bone marrow smears.

Comparison of findings from smears of clinical patients and cases submitted to autopsy about 18 h after death raises the question what change caused by autolysis took place in the chemical composition of the cytoplasm of erythroblasts after death. Bone marrow could be obtained two days before death and during the autopsy 18 h thereafter from a clinical patient with fresh thrombosis of the right arteria renalis and left-sided malignant vascular nephrosclerosis. This case showed a high percentage of PAS-positive erythroblasts before death, but it was clearly diminished thereafter even though the chemical composition of the cytoplasm was not qualitatively changed. On the basis of this observation it can be assumed that in the autopsy cases examined by us larger numbers of PAS-positive erythroblasts were present immediately before death than actually found 18 hours after death.

In order to clarify more precisely the kidney diseases of clinical patients, a kidney biopsy was occasionally carried out in addition to the usual clinical examination methods. In the autopsy cases the extent and degree of kidney damage was judged macroscopically and microscopically by examining paraffin sections of the kidneys in hematoxylin-eosin stain, PAS stain and in elastica-van Gieson stain.

Results

Control Group

The results of examinations of erythroblasts from autopsy cases with hematological and other organic diseases that did not affect the kidneys are shown in Table I. Of them only one case with pancytopenia (case 2) shows 6.5 % PAS-positive erythroblasts on the bone marrow smear. The index is also 6.5. None of the remaining cases examined post mortem reveals PAS-positive substances in the cytoplasm of its erythroblasts.

Among 8 clinical patients with hematological and other organic diseases not affecting the kidneys, 3 who suffered from chronic lymphadenosis (case 8), Morbus BOECK (case 11) and panmyelophthisis (case 13) show PAS-positive erythroblasts in the bone marrow. The percentages lie between 14 % and 0.9 %, just as the index is between 14 and 0.9 (Table I). A relationship between the severity of anemia and the heigth of the percentages of PAS-positive erythroblasts cannot be established. No PAS-positive erythroblasts are found in cases of malignant reticulosis, purpura of unknown pathogenesis, Morbus WERLHOF, ornithosis and of cardiac infarct accompanied by fatty liver.

Results of morphologic and qualitative cytochemical analyses show that the PAS-positive material in the cytoplasm of polychro-

Table I

Control group. Autopsy cases and clinic patients. No PAS-positive erythroblasts on bone marrow smears in diffe organic diseases not affecting the kidneys. Small percentages of PAS-positive erythroblasts in several hematol diseases.

Case	Diagnosis	Hemoglobin g	PAS-pos. erythroblasts %	Ind
	Patho-anatomical			
1	Paramyeloblastic leukemia, leukemic infiltrations of the kidneys, hemorrhagic diathesis. Autopsy-Nr. 168/63	11.9	0	0
2	Pancytopenia, hemorrhagic diathesis. Autopsy-Nr. 43/65	3.8–6.1	6.5	6.5
3	Pancytopenia, pneumonia, septicopyemia. Autopsy-Nr. 149/63	9.2	0	0
4	Lymphosarcoma with sclerosing infiltrations in liver and lymph-nodes, cytostatic treatment. Autopsy-Nr. 58/65	11.3	0	0
5	Diffuse hemorrhage in the intestinal canal. Autopsy-Nr. 312/65	16.0	0	0
6	Bronchial carcinoma, multiple metastasis in bones and parenchymatous organs except the kidneys. Autopsy-Nr. 642/65	7.2	0	0
7	Rupture of an arteria (accident) hemorrhage. Autopsy-Nr. 33/65	11.1	0	0
	Clinical			
8	Chronic lymphadenosis	8.6	0.9	0.9
9	Malignant reticulosis	7.5	0	0
10	Purpura of unknown pathogenesis	14.2	0	0
11	Morbus Boeck	13.5	2.8	2.8
12	Morbus Waldenström	13.6	0	0
13	Panmyelophthisis	6.6	14	14
14	Ornithosis, emphysema	12.0	0	0
15	Cardiac infarct, essential hyperlipemia, fatty liver	13.8	0	0

matic and oxyphilic erythroblasts consists exclusively of glycogen in all cases in the control group. Cytoplasmic distribution of the PAS-positive material is in part diffusely homogeneous and in part granulous.

Kidney Diseases

Table II contains the findings from 13 autopsy cases of different kidney diseases. The number of PAS-positive erythroblasts is related to the macroscopical and microscopical findings from kidneys as well as to the hemoglobin values, blood urea nitrogen or nonprotein nitrogen values respectively and to the creatinine values in the serum. The number of PAS-positive erythroblasts in bone marrow smears fluctuates between 55 % and 10 %. The index lies between 67 and 10. The results show that there is no definite relationship between the severity of anemia and the number of PAS-positive erythroblasts examined post mortem, as those cases of severe anemia prove. Thus, in case 1 (bilateral acute ascending

Table II

Autopsy cases with kidney diseases. High percentages of PAS-positive erythroblasts on bone marrow smears. No definite relationship between the number of PAS-positive erythroblasts and the severity of anemia or the increase in blood urea nitrogen or nonprotein nitrogen respectively as well as creatinine in the serum.

Case	Patho-anatomical diagnosis	Hemoglobin g%	Blood urea nitrogen mg%	Nonprotein nitrogen mg%	Creatinine mg%	PAS-pos. erythroblasts %	Index
1	Bilateral acute ascending pyelonephritis with abscesses. Autopsy-Nr. 234/65	8.6	—	—	5.5	55	67
2	Distinct left-sided chronic sclerosing glomerulonephritis, right-sided renal aplasia. Autopsy-Nr 32/65	6.3	—	129.0	25.1	52	59
3	Complications after plastic surgery of the aorta abdominalis: fresh thrombosis of the right arteria renalis, total infarct of the right kidney, partial infarct of the left kidney; death 5 days post operationem. Autopsy-Nr. 56/65	—	118.0	—	—	41	63
4	Amyloidosis of the kidneys with slight secondary contraction. Autopsy-Nr. 42/65	12.5	—	159.0	6.0	30	41
5	Right-sided acute ascending pyelonephritis with abscesses, operative defect of the left kidney. Autopsy-Nr. 233/65	10.4	130.0	—	—	25	45
6	Distinct benign vascular nephrosclerosis, slight tubular damage. Autopsy-Nr. 32/65	9.8	—	59.0	1.1	23	26
7	Distinct chronic sclerosing glomerulonephritis. Autopsy-Nr. 192/65	5.3	—	198.0	17.3	21	27
8	Distinct benign vascular nephrosclerosis, slight tubular damage. Autopsy-Nr. 38/65	8.2	—	64.2	—	19	19
9	Moderate benign vascular nephrosclerosis, slight tubular damage. Autopsy-Nr. 74/65	12.4	—	65.0	7.1	15	22
10	*Phenacetin poisoning: interstitial nephritis*, necrosis of the renal papillae, moderate benign vascular nephrosclerosis. Autopsy-Nr. 351/65	6.4	310.0	—	16.4	15	26
11	Goodpasture syndrome: subacute glomerulonephritis, pulmonal hemorrhage. Autopsy-Nr. 411/63	6.0	340.0	—	19.0	15	16
12	Bilateral acute ascending pyelonephritis with abscesses. Autopsy-Nr. 641/65	11.5	—	100.5	—	14	14
13	Subacute glomerulonephritis. Autopsy-Nr. 185/65	7.0	—	96.0	8.15	10	10

pyelonephritis with abscesses) 55% PAS-positive erythroblasts and 8.6% hemoglobin is found, in case 13 (subacute glomerulonephritis) 10% PAS-positive erythroblasts and 7 g% hemoglobin. Furthermore, there is no definite relationship between the increase in blood urea nitrogen or nonprotein nitrogen respectively and in creatinine in the serum—tests which were taken as a measurement of the distorted function of the kidneys—and the percentage of PAS-positive erythroblasts. Hence, case 4 (amyloidosis of the kidneys with slight

Table III

Clinic patients with kidney diseases. High percentages of PAS-positive erythroblasts on bone marrow smears. No definite relationship between the number of PAS-positive erythroblasts and the severity of anemia or the increase of blood urea nitrogen as well as creatinine in the serum.

Case	Diagnosis	Hemoglobin	Blood urea nitrogen mg %	Creatinine mg	PAS-pos. erythroblasts %	Index
1	Acute kidney failure	12.2	382	13.0	52	100
2	Left-sided malignant nephrosclerosis, fresh thrombosis of the right arteria renalis (Autopsy)	16.0	305	10.0	33	43
3	Acute kidney failure (biopsy)	7.2	340	15.0	30	22
4	Amyloidosis of the kidney	14.0	60	2.0	25	25
5	Chronic glomerulonephritis	7.0	512	17.0	25	30
6	Chronic ascending pyelonephritis	9 6	380	8.0	24	24
7	Polycystic kidney	10 0	330	13.0	16.8	16
8	Chronic ascending pyelonephritis	13.0	56	2.2	13	14
9	Chronic ascending pyelonephritis	13.0	90	3 7	11	12

secondary contraction) shows 30 % PAS-positive erythroblasts and 159 mg % nonprotein nitrogen and 6 mg % creatinine, case 11 (Goodpasture syndrome) 15 % PAS-positive erythroblasts and 340 mg % blood urea nitrogen and 16 4 mg % creatinine. On the other hand *there is a relationship* between the patho-anatomical findings from kidneys and the number of PAS-positive erythroblasts. Those cases with the highest number of PAS-positive erythroblasts are the ones in which considerable loss of renal parenchyma or diseases of tubules are observed, as for example (Table II) chronic sclerosing glomerulonephritis (cases 2 7) benign vascular nephrosclerosis (cases 6 8) thrombosis of both arteriae renales (case 3) amyloidosis of the kidneys (case 4) and lastly severe acute ascending pyelonephritis (cases 1 5 12) Smaller numbers of PAS-positive erythroblasts are found in subacute glomerulonephritis (case 13) Goodpasture syndrome (case 11) phenacetin poisoning with chronic interstitial nephritis (case 10) and moderate benign vascular nephrosclerosis (case 9)

Table III contains the cytochemical and serological findings from 9 patients suffering from kidney diseases. The percentage of PAS-positive erythroblasts in the bone marrow smears fluctuates between 52 and 11 % the calculated index between 100 and 12. Here too there is no definite relationship between the severity of anemia and the percentage of PAS-positive erythroblasts on bone marrow smears Thus, case 1 (acute kidney failure) shows 52 PAS-

positive erythroblasts and 12.2 g% hemoglobin and case 9 (chronic ascending pyelonephritis) 11% PAS-positive erythroblasts and 13 g% hemoglobin. Just as in those cases mentioned in Table II these cases also do not indicate any definite relationship between the increase in blood urea nitrogen and creatinine in the serum and the percentage of PAS-positive erythroblasts Thus, in case 4 (amyloidosis of the kidneys) 25 % PAS-positive erythroblasts and 60 mg% blood urea nitrogen as well as 2 mg% creatinine are found and in case 7 (polycystic kidneys) 16.8 % PAS-positive erythroblasts and 330 mg% blood urea nitrogen as well as 13 mg % creatinine. However it can be established that patients with a so-called acute failure of the kidneys or nephrosclerosis as well as patients with amyloidosis of the kidneys show the highest percentage of PAS-positive erythroblasts (cases 1 through 5 Table III)

Furthermore, it is important to clarify whether or not PAS-positive material in the cytoplasm of erythroblasts can be found in patients with slightly to moderately severe damage of tubules in the kidneys. In two cases (autopsy case and clinical patient) a myeloma was present and in another autopsy case a Morbus WALDENSTROEM. Proximal nephron nephrosis could be found clinically and histologically in all three cases. Two other autopsy cases revealed cholemic nephrosis, in one case due to lymphogranulomatosis infiltrating the liver and the kidneys slightly and in the other case because of a carcinoma in the gall bladder with metastasis in liver and bones. In all five cases a slight to moderate increase in the number of PAS-positive erythroblasts is found. The percentages fluctuate between 6.8 and 5 %. The index is close to 6, the hemoglobin between 7 g% and 10 g%.

Cytochemical Examinations of the Erythroblasts

Results from morphological and qualitative cytochemical analyses of the PAS-positive substance in the cytoplasm of erythroblasts in patients with kidney diseases show that the PAS-positive material is particularly distributed diffusely and homogeneously excluding a fine perinuclear area in the cytoplasm (Fig 1) Sometimes granulous PAS-positive substances can be found in individual erythroblasts too. The PAS-positive erythroblasts belong to the maturity stages—judged by the shape of the nucleus and the width of cytoplasm—of polychromatic and oxyphilic erythroblasts. Table IV shows the results from the most important histochemical reac

Table II

Autopsy cases with kidney diseases. The most important cytochemical findings in the cytoplasm of erythroblasts o bone marrow smears. The PAS-positive material consists of glycogen, lipids and glycoproteids or mucoproteids re spectively or neutral mucopolysaccharides too and very small amounts of acidic mucopolysaccharides and DNA

Autopsy Nr	Erythroblasts in Percent in PAS	PAS after saliva	PAS after lipid extraction	Ninhydrin Schiff	Millon	Toluidine blue	Feulgen
254/63	55	50	16	40	25	1	—
32/63	52	—	—	—	—	—	—
56/65	41	6.5	7.7	33	40	1.7	0.4
42/63	30	7.1	22.5	59	—	8.5	2.8
253/65	23	12	3.8	46	26	2	—
5_/65	23	5	9	25	15	6	2.1
197/63	21	8	0.2	60	31	0	0
38/65	19	4	14.7	—	—	1.5	—
74/65	15	1	9	29	44	1	—
351/65	15	12	15	58	18	1	—
414/65	15	5	4	21	20	2	—
641/65	14	6	10	—	30	0	0
185/65	10	10	2.6	68	34	0	0



Fig. 1. Bone marrow smear in kidney disease. PAS stain. PAS-positive (with black cytoplasm on the photo) and PAS-negative erythroblast (about 1600).

tions of the erythroblasts from autopsy cases. Cytochemical examinations of erythroblasts of clinical patients give the same results. The differentiation of the PAS-positive material using saliva treatment, lipid extraction and then PAS staining as well as Best's carmine staining and sudan *black B* staining shows *that both glycogen* and lipids (Fig. 2) are present. PAS staining after acetylation and deacetylation indicates the presence of 1 2-glycols. The presence



Fig. 2. Bone marrow smear in kidney disease. Sudan black B stain. Left upper corner erythroblast *with sudanophilic granules in the cytoplasm, right and left lower corner* myeloblasts with sudanophilic granules (about ×2250).



Fig. 3. Bone marrow smear in kidney disease. Toluidine blue stain. Erythroblast with metachromatic (on the photo black) granules in the cytoplasm (about ×2250).

of very slight amounts of granulous metachromatic substances can be shown with toluidine blue staining (Fig. 3) which lead to the assumption of acidic mucopolysaccharides being present. However FEULGEN's stain indicates very slight amounts of DNA too. After treating the smears with saliva and then lipid extraction a relatively weak pink but easily identifiable, diffusely homogeneous cytoplasm stain is none the less found in PAS staining of erythroblasts. Positive proof of the presence of free NH-groups and tyrosine is given by ninhydrin-SCHIFF reaction and MILLON's reaction. Therefore the remaining positive PAS stain in the erythroblasts after combined treatment of saliva and lipid extraction indicates the presence of glycoproteids or mucoproteids respectively or neutral mucopolysaccharides too. Further cytochemical differentiation of these three substances is not possible with the methods available today.

Discussion

Our results show on bone marrow smears of patients and dead with renal diseases accompanied by anemia that a high percentage of polychromatic and oxyphilic erythroblasts contains PAS-positive material. Qualitative cytochemical analyses of this PAS-positive material show—for both patients and the dead—that it consists partially of glycogen and lipids, partially of glycoproteids or mucoproteids respectively or neutral mucopolysaccharides too. Very slight amounts of acidic mucopolysaccharides and granules containing DNA are present in the cytoplasm. On the other hand erythroblasts of healthy persons do not show PAS-positive material in their cytoplasm (1 20 30 34) We also are not able to find PAS-positive erythroblasts on bone marrow smears of patients who suffered or died from diseases that neither affected the kidneys directly nor indirectly A few hematological diseases are exceptions.

The presence of PAS-positive material in the erythroblasts is dependent on the degree to which kidney parenchyma is lost even though no definite relationship exists between the elevation of blood urea nitrogen and creatinine in the serum and the number of PAS-positive erythroblasts in the bone marrow Thus, the highest percentage of PAS-positive erythroblasts is found in chronic sclerosing glomerulonephritis and in benign and malignant vascular nephrosclerosis, in thrombosis of both arteriae renales and in amyloidosis of the kidneys (Table II and III) In addition we are able to prove that a small amount of PAS-positive erythroblasts can be found in diseases like myeloma and Morbus WALDENSTROEM which form a pathological protein secreted by the kidneys and show tubular damage histologically as well as in diseases which lead to cholemic nephrosis too

In some hematological diseases PAS-positive material can appear in the cytoplasm of erythroblasts. ASTALDI *et al* (2) were the first to point to this pathological material in polychromatic and oxyphilic erythroblasts in thalassaemia. Later other researchers confirmed these findings (15 30 and others) Cytochemical analyses of these erythroblasts show that only glycoproteids or mucoproteids respectively or neutral mucopolysaccharides too are present in this PAS-positive material (3)

A high percentage of PAS-positive erythroblasts is also found in erythroleukemia, myelosis erythraemica acuta (Morbus Di Guglielmo) and in sideroachrestic anemia (4 5 19 25 35) The same is also true of other hematological diseases like pernicious anemia (25 35) aplastic anemia (25) polycythemia (25) pyridoxine responsive anemia (4) osteomyelosclerosis (30) several types of leukemia (4 30) and a few types of hemolytic anemia (30) In all of these diseases the PAS-positive material in the cytoplasm of the erythroblasts proved to be glycogen (21 25 30) Furthermore, PAS-positive erythroblasts are found in cases of anemia after lead poisoning (21) and after benzol poisoning (14) Ferrara (14) observed that the PAS-positive material cannot be removed enzymatically with saliva after benzol poisoning Whether or not the deposition of PAS-positive material is effected by tubular damage in the kidneys after these poisonings remains an open question.

Our examinations also show slight numbers of PAS-positive erythroblasts on sternal bone marrow smears of patients with pancytopenia and panmyelophthisis, chronic lymphadenosis as well as Morbus Boeck. Cytochemical analyses of this PAS-positive substance show it to be glycogen exclusively

Thus, according to our examinations, the pathological substances in the cytoplasm of the erythroblasts of hematological diseases differ cytochemically from those found in kidney diseases In kidney diseases glycogen, lipids, glycoproteids or mucoproteids respectively or neutral mucopolysaccharides too and acidic mucopolysaccharides as well as DNA can be found. In contrast, in hematological diseases only glycogen is present.

Whether or not the development of anemia nd the presence of PAS-positive material in the erythroblasts of patients with loss of renal parenchyma or nephrosis are effected only by uremia or by insufficient formation or even loss of a material that is formed in the kidneys and influences the chemical composition of the cytoplasm of erythroblasts, this is a question which cannot be answered from the present findings. It is, however striking that there is no definite relationship between the amount of blood urea nitrogen and creatinine in the serum—as a measurement of the degree of distorted kidney function—and the number of pathologically changed erythroblasts.

In order to clarify this problem experiments were carried out on rats with bilateral nephrectomy and rats with bilateral ureter ligation. Rats with bilateral ureter ligation and severe uremia did not show an increased number of PAS-positive erythroblasts on bone marrow smears at the time of death, whereas rats with bilateral nephrectomy showed a high percentage of PAS-positive erythroblasts at death. Cytochemical analysis of this PAS-positive material revealed the same chemical composition as in the erythroblasts from patients with kidney diseases. If the nephrectomized rats were injected daily post operationem with rat kidney homogenates PAS-positive erythroblasts could no longer be found in the bone marrow smears at death. Injection of adequate amounts of human kidney homogenates into nephrectomized rats had the same effect, whereas injection of homogenates from rat spleen or liver was not successful. Results from experimental examinations carried out until now permit the conclusion that a material is formed in the kidneys of rats and men which has influence on the chemical composition of the cytoplasm of erythroblasts. If the results of these animal experiments can be assumed to be valid in human biology too it would mean that the pathological changes of the chemical composition of human erythroblasts in kidney diseases are caused by either reduced formation or even loss of this renal material. A detailed discussion of these experimental data will be given in the near future.

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Summary

PAS-positive erythroblasts are found in the bone marrow of patients with kidney diseases. The number of PAS-positive erythroblasts is dependent on the degree of renal parenchyma loss or the severity of the tubular damage. There is no definite relationship between the severity of anemia and the number of PAS-positive erythroblasts. Qualitative cytochemical analysis of these PAS-positive polychromatic and oxyphilic erythroblasts show glycogen and lipids as well as glycoproteids or mucoproteids respectively or neutral mucopolysaccharides too and small amounts of acidic mucopolysaccharides and DNA in the cytoplasm. Experiments on bilateral nephrectomy and bilateral ureter ligation in rats lead to the assumption that a material is formed in the kidney of men and rats which influences the chemical composition of the cytoplasm of erythroblasts.

Zusammenfassung

Im Knochenmark von Patienten mit Nierenkrankheiten finden sich PAS-positive Erythroblasten. Ihre Zahl hängt vom Ausmaß des Verlustes an Nierenparenchym oder von der Schwere der Tubulusschädigung ab, sie steht jedoch nicht in Beziehung zur Schwere der Anämie. Die qualitative zytochemische Analyse dieser PAS-positiven polychromatischen und oxyphilen Erythroblasten ergibt Glykogen und Lipide, sowie Glykoproteide oder Mukoproteide oder neutrale Mukopolysaccharide und kleine Mengen von sauren Mukopolysacchariden und DNA im Zytoplasma. Versuche mit bilateraler Nephrektomie und mit bilateraler Ureterligatur bei Ratten lassen annehmen, daß in der Niere von Mensch und Ratte eine Substanz gebildet wird, die die chemische Zusammensetzung des Zytoplasmas der Erythroblasten beeinflußt.

Résumé

Dans la moelle osseuse de malades atteints de maladies rénales se trouvent des érythroblastes PAS-positifs. Leur nombre dépend de la perte en parenchyme rénal ou de la gravité de l'atteinte tubulaire, mais n'est cependant pas en relation avec le degré de l'anémie. L'analyse cytochimique qualitative de ces érythroblastes PAS-positifs et oxyphiles met en évidence dans le cytoplasme du glycogène et des lipides, ainsi que des glycoprotéides ou des mucoprotéides, ou encore des mucopolysaccharides neutres, de petites quantités de mucopolysaccharides acides et de l'ADN. Les résultats d'expériences faites sur des rats, néphrectomies bilatérales et ligatures des uretères, laissent supposer qu'une substance est formée dans les reins de l'homme et du rat, substance qui influence la composition chimique du cytoplasme des érythroblastes.

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Die Lokalisation der unspezifischen Esterase in foetalen Erythrozyten und deren Vorstufen

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Durch eine Reihe von physikalischen und färberischen Methoden wurden Einzelstrukturen in den Erythrozyten und ihren Vorstufen nachgewiesen, ohne daß es immer gelang, die Einzelbefunde der verschiedenen Methoden miteinander abzustimmen oder gar zur Deckung zu bringen. Daher waren Kombinations- oder Succedanfärbungen willkommene Verfahren, um scheinbar beziehungslose Strukturen als identisch und tatsächlich verschiedenartige Zellbestandteile als different zu erkennen. Ein solches Verfahren teilte Bearric (5) mit. Er stellte in einer kombinierten Färbung basophile Tüpfelung und Siderocyten-Granula gleichzeitig dar und bewies damit ihre grundsätzliche Verschiedenheit. Rind und Stobbe (45) konnten phasenpositive Granula im Normoblasten und Retikulozyten mit Janusgrün als Mitochondrien identifizieren. Thompson (51) gelang es, Heinz'sche Innenkörper und Substantia granulofilamentosa simultan darzustellen. Bessis und Breton-Gorius (6) sowie Staubesand et al. (48) stellten systematische Vergleiche von Supravitalfärbungen, Phasenkontrast und Elektronenmikroskopie des Proerythrozyten und Erythrozyten an.

Ungeklärt war es bisher, welche Beziehungen zwischen der erstmals von Wachstein und Wolf (52) beschriebenen zytochemisch nachweisbaren Alpha-Naphthyl-Acetat-Esterase („unspezifische Esterasen") in den Erythroblasten und demselben von Davis (11) in Erythrozyten nachgewiesenen Ferment bestehen; noch viel weniger war bekannt, an welche Zellstrukturen diese Fermentaktivitäten gebunden sind.

Die hier vorgelegten Beobachtungen am Blut von menschlichen Foeten und Neugeborenen geben Hinweise auf die engen Beziehungen von plasmatischer Esterase-Aktivität, kernhaltiger und

kernloser roter Blutzellen. Daraufhin angestellte Experimente sollten klären, wie sich die mit Supravitalfarbstoffen darstellbaren Strukturen zu den fermentzytochemisch erfaßten Gebilden verhalten. Und zwar wurde versucht, beide Reaktionen an jeweils derselben Blutzelle nacheinander vorzunehmen und auch mikrophotographisch festzuhalten.

Material und Methoden

Es wurde von reifen Neugeborenen unmittelbar nach der Abnabelung Blut aus dem placentaren Teil der Nabelschnur entnommen. Desgleichen wurde aus den Herzhöhlen frischtoter Neugeborener und von menschlichen Foeten bis herab zu Embryos von 7 cm Scheitel-Fersen-Länge Blut gewonnen. Für die Pappenheim-Färbung und die einfache Fermentreaktion wurden dünne Blutausstriche angefertigt.

1. Fermentreaktion. Zur zytochemischen Darstellung der Alpha-Naphthyl-Acetat-Esterase eignet sich die von D…n (11) angegebene Methode in einer Modifikation von LEDER und NICOLAS (33). J… ein Tropfen Pararosanilin und 4 …iges Natriumnitrit wurde eine Minute gemischt, danach 25 ml 0,1 M Phosphatpuffer pH 7,5 sowie 10 mg Alpha-Naphthyl-Acetat, gelöst in 0,8 ml Aceton, hinzugefügt. Die luftgetrockneten Präparate wurden für eine Stunde inkubiert, … unter Leitungswasser abgespült und die Kerne mit Hämalaun gegengefärbt, sodann in Gelatinol eingedeckt. Fermenthaltige Strukturen wurden leuchtend rotbraun in exakter Lokalisation dargestellt.

2. Supravitalfärbung. Die Farbstoffe wurden grundsätzlich in physiologischer Kochsalzlösung gelöst: 1% Brillantkresylblau, 0,12 … Janusgrün und 0,5 … Nilblausulfat wurden jeweils im Verhältnis 1 : 4 mit Blut gemischt, Färbung bis zu 30 Minuten in einer feuchten Kammer. Im Anschluß daran wurden dünne Blutausstriche angefertigt.

3. Simultandarstellung. Die supravital gefärbten Präparate wurden mit Immersionsobjektiv photographiert, in Xylol abgewaschen und der Fermentreaktion unterzogen. Bei diesem Vorgehen ist lediglich das …olle Mikroskopierlicht in der Lage, nach Einwirkung von nur wenigen Minuten Dauer die Fermentaktivität zum Verschwinden zu bringen. Sie konnte jedoch durch zwischengeschaltete UV- und Wärmeschutzfilter und durch möglichst geringe Beleuchtung des Präparates erhalten …rden.

Ein Auswaschen der Supravitalfarbstoffe erübrigte sich, da sie sich in dem wäßrigen Inkubationsgemisch der Fermentreaktion … eingehend wieder … aus der Zelle herauslösten.

Ergebnisse

Im peripheren Blut Neugeborener finden sich vermehrt Proerythrozyten (2, 37) und regelmäßig Normoblasten. Daher sind in einem Blutausstrich auch alle Stadien der Kernausstoßung zu finden. Je weiter wir die Embryonalentwicklung zurückverfolgen, desto häufiger treffen wir neben den normalen Erythroblasten auf abnorme Bildungen mit bis zu 8 Kernen oder Kernabschnürungen (44).

Wie bei Knochenmarks-Erythroblasten (32, 38, 34) läßt sich auch in den Erythroblasten des peripheren foetalen und Neugeborenen-Blutes regelmäßig unspezifische Esterase nachweisen, ebenso

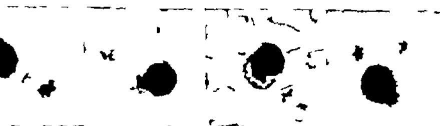


Abb. 1 Kernausstoßungen im Blute eines Frühgeborenen. Unspezifische Esterase-Reaktion. Die fermentaktive Substanz ordnet sich hinter dem Kern an (1400 x)



Abb. 2 Proerythrozyten mit stark esterasepositiven Zentren nach der Kernausstoßung Frühgeborenenblut (1400 x)

wie in embryonalen Zellen der »mesenchymalen« oder »megaloblastischen« Bildungsperiode (7) die nach KÜNZER (30) mit den Megaloblasten der Perniciosa verwandt sein sollen Dabei stellt sich ein den Kern gleichmäßig umgebender fermentaktiver Saum dar Während die Entkernung der lebenden Zelle bei phasenoptischer Beobachtung unter extremen Plasmakontraktionen vor sich geht (45) sehen wir im Ausstrichpräparat lediglich eine abgerundete oder ovale Zelle oft mit einer besonders schlaff erscheinenden Membran, worin der Kern aus der Mitte an die Peripherie verlagert ist und in einigen Fällen durch die Zellmembran hindurch tritt. In allen diesen Fällen ordnet sich die fermentpositive Substanz in charakteristischer Weise hinter dem Kern in der Zellmitte an (Abb 1) In den mehrkernigen Erythroblasten sammelt sie sich regelmäßig zwischen den Kernfragmenten an Der ausgestossene Kern zeigt eine mittlere Fermentaktivität, während die vorher perinucleär gelegene Fermentansammlung zentral in der entkernten Zelle liegen bleibt. Durch diese Beobachtung lassen sich die esterase positiven Körper der kernlosen roten Zellen direkt von den perinucleär liegenden Substanzen des Normoblasten ableiten die kernlosen Zellen mit fermentaktiven zentralen Ansammlungen (Abb 2) sind somit als Proerythrozyten definiert. Besonders in den

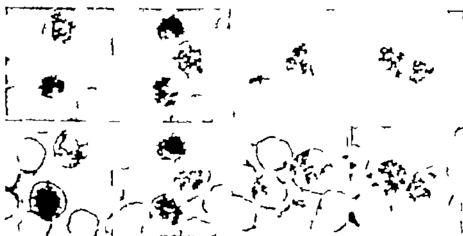


Abb. 3. Obere Reihe: Supravitalfärbung mit Brillantkresylblau. Proerythrozyten verschiedener Reifestufen.
Untere Reihe: Dieselben Zellen nach anschließender Esterasereaktion; die Substantia granulofilamentosa erscheint fermentpositiv. In den jüngeren Proerythrozyten kräftiger fermentaktiv. Granula. Frühgeborenenblut (1400 ×)

größeren der zentralen Fermentansammlungen finden sich außerdem einzelne, sehr kräftig leuchtende fermentaktive Granula (»zentrale Granula«). Weiterhin erscheinen in einem großen Teil der reifen Erythrozyten ein oder wenige, meist nahe dem Zellrand gelegene scharf begrenzte und kräftig leuchtende Körnchen (»periphere Granula«).

Welche Beziehungen zwischen diesen Zellelementen und der Substantia granulofilamentosa der Proerythrozyten bestehen, läßt sich durch aufeinanderfolgende Darstellungen bestimmen. In der Supravitalfärbung mit Brillantkresylblau stellt sich die Substantia granulofilamentosa in der bekannten Weise blauschwarz dar. Dasselbe Netzwerk zeigt bei nachfolgender Fermentreaktion eine kräftige Aktivität (Abb. 3–5). Der kompakt-strahlige Körper der direkten Fermentreaktion ist somit bei vorhergehender Brillantkresylblaufärbung in die netzförmige Struktur überführt worden. In den Retikulozyten-Reifestufen I und II (19, 20) finden sich die ihm zugehörenden zentralen Granula innerhalb des kräftig leuchtenden Netzwerkes; in den Reifestufen III und IV erscheinen sie selten, und die Fermentaktivität der Filamente ist deutlich schwächer.

Eine nähere Bestimmung dieser Granula gelingt mit der Janusgrün-Färbung. Hier stellen sich meist zentral liegend bis zu etwa

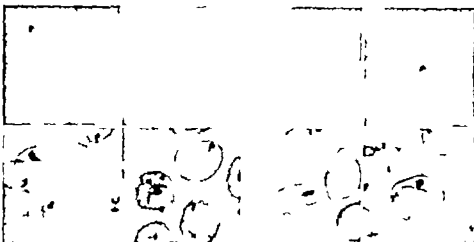


Abb 4 Obere Reihe Supravitalfarbung mit Janusgrün, Darstellung von Mitochondrien. Untere Reihe Dieselben Zellen nach anschließender Fermentreaktion. Kräftige Reaktion im Bereich der Mitochondrien. Frühgeborenenblut (1400).

10 Mitochondrien in einer Zelle dar Bei der anschließenden Fermentreaktion erscheinen die fermentaktiven Granula an der Stelle der Mitochondrien (Abb 4 5) Die größer erscheinenden Azofarbstoff Präzipitate lassen jedoch nur eine geringere Zahl von Granula optisch voneinander getrennt erscheinen als der Supravitalfarbstoff

Von den peripher liegenden fermentaktiven Granula, die sich im foetalen und Neugeborenen-Blut in einem hohen Prozentsatz der Erythrozyten finden wird ein meist kleiner Anteil durch Brillantkresylblau angefärbt. Ihre auffallende Randlage – haufig scheinen sie direkt an der Zellmembran zu liegen – läßt jedoch an die von verschiedenen Autoren genauso beschriebene Lage der erst mals von Heinz (21) beschriebenen Innenkörper (siehe die Übersicht bei 13) denken Heinz sche Innenkörper färben sich mit basischen Farbstoffen wie Brillantkresylblau und vor allem Nilblausulfat an Supravitalfärbungen von Neugeborenenblut mit diesen beiden Farbstoffen ergeben in beiden Fällen gleiche, meist niedrige Werte von Heinz schen Innenkörpern Läßt man der Supravitalfarbung die Esterase-Reaktion folgen, so erweisen sich alle farbstoffpositiven Heinzkörper als kräftig fermentpositiv aber darüber hinaus erscheinen die fermentpositiven peripheren Granula in der Mehrzahl aller Erythrozyten (Abb 6) In ihrer peripheren,

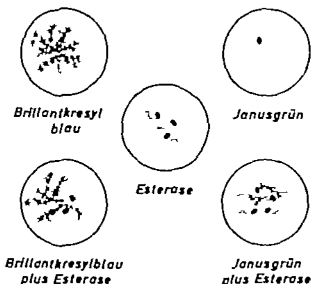


Abb. 5. Schema der Innenstrukturen des Proerythrozyten: oben Supravitalfärbungen der Substantia granulofilamentosa (links) und von Mitochondrien (rechts). Unten die fermentpositiven Strukturen in den vorgefärbten Zellen. In der Mitte eine nicht vorbehandelte Zelle mit ihren fermentpositiven Strukturen. Brillantkresylblau führt zu einer Koagulation der Zellbestandteile, Janusgrün nicht.

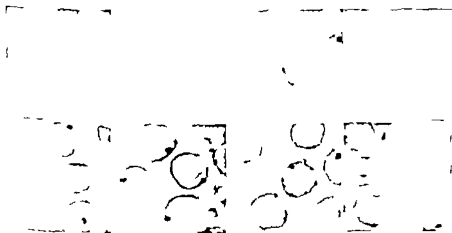


Abb. 6. Obere Reihe: Supravitalfärbung von reifen Erythrozyten mit Nilblausulfat. Darstellung von Heinz'schen Körperchen.
Untere Reihe: Dieselben Zellen nach anschließender Fermentreaktion, die Heinzkörper erscheinen kräftig esterasepositiv. Darstellung von mehr Körperchen als in der Supravitalfärbung.
Frühgeborenenblut (1400)

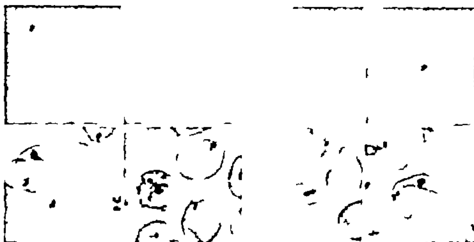


Abb. 4 Obere Reihe Supravitalfärbung mit Janusgrün, Darstellung von Mitochondrien. Untere Reihe Dieselben Zellen nach anschließender Fermentreaktion. Kräftige Reaktion im Bereich der Mitochondrien.
Frühgeborenenblut (1400).

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noch reversible Veränderung während die Anfärbbarkeit ein späteres, irreversibles Stadium der Vergiftung markiert.

Aufgrund dieser Angaben sowie unserer eigenen Befunde halten wir es für wahrscheinlich, daß die zahlreichen peripheren fermentaktiven Granula den elektronenoptisch darstellbaren Körpern entsprechen, von denen ein jeweils verschieden großer Anteil durch Nilblausulfat als HEINZkörper angefärbt werden kann. Ob die nicht anfärbbaren peripheren Körper jüngere Stadien von HEINZkörpern darstellen oder Zeichen einer schwächeren Alteration sind, läßt sich aus unseren Befunden nicht schließen. Da jedoch in einem HEINZkörper reichen Blut die Übereinstimmung von färbbaren mit fermentpositiven Granula im Gegensatz zum HEINZ körper armen Blut hohe Prozentsätze erreicht, scheint jedes nicht anfärbbare fermentpositive periphere Granulum im Erythrozyten ein potentielles HEINZ Körperchen zu sein.

Die positive Fermentreaktion der HEINZkörper weist darauf hin, daß an den Ablagerungsorten der Vergiftungsprodukte auch chemische Vorgänge ablaufen, die möglicherweise mit dem Abbau dieser Stoffe im Zusammenhang stehen.

Zusammenfassung

Die Alpha-Naphthyl-Acetat-Esterase läßt sich zytochemisch in Erythroblasten, Proerythrozyten und Erythrozyten des peripheren foetalen und Neugeborenen-Blutes nachweisen. Mit Hilfe von Succedanfärbungen wird gezeigt, daß die Fermentaktivität im Proerythrozyten an die Substanzen gebunden ist, die bei Einwirken von Brillantkresylblau die Substantia granulofilamentosa bilden. Besonders starke Aktivität findet sich im Bereich der Mitochondrien. Die in reifen Erythrozyten nachweisbare granuläre Esteraseaktivität entspricht den im Neugeborenenblut regelmäßig vorkommenden Heinz'schen Innenkörpern.

Summary

Alpha-naphthyl acetate esterase can be demonstrated by the cytochemical method in erythroblasts, proerythrocytes and erythrocytes of the peripheral blood of the foetus and newborn. It can be shown by succedan staining that the enzyme activity of proerythrocytes is dependent on those substances which under brilliant cresyl blue form the granulofilamentous substance. Particulary marked activity is found in the neighbourhood of the mitochondria. The granular esterase activity found in mature erythrocytes corresponds to the Heinz inclusion bodies regularly present in the blood of newborn infants.

Résumé

L'alpha-naphtyl-acétate-estérase peut être mise en évidence par des méthodes cytochimiques dans les érythroblastes, proérythrocytes et érythrocytes du sang périphérique foetal et dans celui du nouveau-né. A l'aide de colorations successives, il a été

démontré que l'activité enzymatique des proérythrocytes est liée aux structures qui apparaissent à la coloration au crésyl bleu brillant sous la forme de la substance granulo-filamenteuse. *Une activité spécialement forte se trouve à l'emplacement des mitochondries.* L'activité de l'estérase qui peut être démontrée sous forme de granules dans les érythrocytes matures correspond aux inclusions de HEINZ se trouvant régulièrement dans le sang du nouveau-né.

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Studies on the Metabolism of Iron Sorbitol

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Since the discovery of saccharated iron oxide by Nissim (16) parenteral iron treatment has been used extensively but owing to various untoward side effects, a search has been made for more suitable iron preparations. Iron dextran—i.e. dextran of high molecular weight combined with iron—was developed by Fletcher and London (4) in 1954 In numerous subsequent papers it has been reported to have fewer side effects and to be more effective in clinical use (2 5 7) While Richmond (20) and others (8, 9 21) have spoken of carcinogenic properties, other workers have found practically no such side effects in patients treated with iron dextran. The problem in this respect remains controversial.

Recently Lindvall and Andersson (1) introduced an iron sorbitol citric acid complex (iron sorbitol) This compound has a molecular weight not exceeding 5000 and contains 15 6% of iron. Numerous authors report that iron-deficient patients have responded satisfactorily to intramuscular injection of iron sorbitol with few side effects (1 3 19 22, 24) Scarcely any carcinogenic properties have been noted (11)

This paper is concerned with the results of our investigation on the metabolism of iron sorbitol and its clinical effects compared with those of iron dextran as previously reported (14)

Material and Methods

(1) Normal rabbits weighing about 2 kg and maintained on conventional laboratory diet were used. A single dose of ^{59}Fe-labelled iron sorbitol (^{59}Fe-sorbitol, 1.36 µc/50 mg iron) was injected into gluteal muscle or ear vein in dose equivalent to 10 mg iron per kg body weight. Two or three animals were sacrificed by perfusion with normal saline at the following times after injection 24 h and 3 7 and 14 days. Blood samples

were taken from the femoral artery immediately before sacrifice. The total urine excreted from the time of injection until sacrifice was collected. Various organs were homogenized, and radioactivity was determined with a well-type scintillation counter. The total radioactivity in blood, urine and each organ was calculated from the following formula:

Total radioactivity (cpm) = cpm/g of specimen in wet weight × total wet weight (g).

The total weight of the bone marrow was taken as 1.5% of the body weight (19). The percentage of radioactivity in each organ, blood and urine, relative to the injected dose, was recorded. Controls were injected with ^{59}Fe-labelled iron dextran (^{59}Fe-dextran, 2.2 μc/90 mg iron) in a dose equivalent to 10 mg of iron per kg body weight.

(2) ^{59}Fe-sorbitol in a dose equivalent to 90 mg of iron (specific activity 3.36 μc per 50 mg iron) was injected intramuscularly into 3 patients with iron deficiency anaemia. Blood samples were taken at selected times from 180 min to 13 days following injection. At each sampling the radioactivity in plasma and whole blood was measured separately by means of a well-type scintillation counter. The blood volume was determined by dye dilution method (6). The total plasma and red-cell activity levels at each sampling time were calculated and expressed as ratios of the dose injected. The radio-iron content of urine collected at 0–3, 3–6, 6–12 and 12–24 (or –48) h following injection was determined.

(3) Clinical effects of iron sorbitol were observed in 12 patients with iron deficiency anaemia. Seven patients received iron sorbitol equivalent to 400 mg iron weekly and the other 5 cases 700 mg weekly. When, however, some of the latter received a daily dose of 200 mg iron, various signs of iron intoxication occurred; the subsequent daily dosage was therefore limited to 100 mg. The total amounts of iron required were calculated from the following formula:

Required iron (mg) = W [2.7(16–A) + 17], where W is the body weight (kg) and A the haemoglobin level (g/dl). Iron sorbitol was injected alternately into the right and left gluteal muscle until 50% in excess of the calculated required dose was attained, taking into consideration urinary excretion. In 3 cases, 24-hour urine was collected daily or at irregular intervals during treatment. The iron content of the urine and serum was determined by a modification of Landers and Zak's method (10, 15). The unsaturated iron binding capacity (UIBC) was determined by Peters's method (18).

Results

(1) Animal Experiments

(a) Distribution of Radio-Iron after a Single Intramuscular Injection of ^{59}Fe-Sorbitol

The distributions of ^{59}Fe in various organs of rabbits at different times following injection of ^{59}Fe-sorbitol or ^{59}Fe-dextran are shown in Fig. 1. The mean radioactivities for each organ (cpm/g wet weight) are listed in Table I. Significant differences between iron sorbitol and iron dextran were noted with respect to residual radioactivity at the injection site and the amount excreted via the kidneys. Iron sorbitol disappeared rapidly from the injection site, more than 60% of the injected dose being excreted in the urine, whereas iron dextran cleared slowly from the site of injection and no excretion

Table I

Concentration of ^{59}F various organs after single injection of ^{59}F -sorbitol.

	Concentration of F (cpm/g tissue) Intramuscular injection after 1	3	7	14 days	Intravenous injection after 1	3	7	14 days
Bone marrow	141	832	389	238	515	300	109	551
Blood	62	534	496	272	66	127	346	506
Liver	946	1 274	1 366	867	873	878	636	408
Spleen	262	5 461	317	123	241	161	171	772
Kidney	942	690	715	176	706	766	354	715
Lymph node								
adjacent to inj. site	10 300	6 770	5 302	4145				
Iliac	1 345	12 200	12 020	5580	610	580		0
Thoracic	10 690	4 399	12 830	1900	0	3065		
Mesenteric	1 025	763	3 873	39	1220	231	28	143
Peri-aortic			4 140	216				
Axillar					280	1725		0

Table Ib

Concentration of ^{59}F in various organs after single injection of ^{59}Fe-dextran.

	Concentration of F (cpm/g tissue) Intramuscular injection after 1	7	14 days	Intravenous injection after 1	7	14 days
Bone marrow	2 716	2 970	3 730	3097	3730	2839
Blood	344	446	668	169	518	320
Liver	751	383	132	3480	4353	2432
Spleen	742	362	132	2470	3510	2350
Kidney	54	113	121	136	142	155
Lymph node adjacent to inj. site	40 117	79 750	15 700			
Iliac	45 125	123 500	26 400			
Thoracic	11 900	28 393	50 100	379		1178
Mesenteric		83	114			379
Peri-aortic	69 100	36 375	4 480			1499
Inj. site	3 950	2 559	2 470			

was detectable in the urine. Thus, there was a marked difference in organ distribution. Within 2 weeks about 8 to 15 /₀ of the injected iron had been utilized for haemoglobin formation in the case of iron sorbitol, and about 20% in the case of iron dextran. After single injections, changes in radioactivity in liver and bone marrow were less pronounced for iron sorbitol than for iron dextran. The renal distribution 24 h after injection was high for the former and very low for the latter It is noteworthy that with both substances a higher radioactivity was found in the lymph nodes than in other

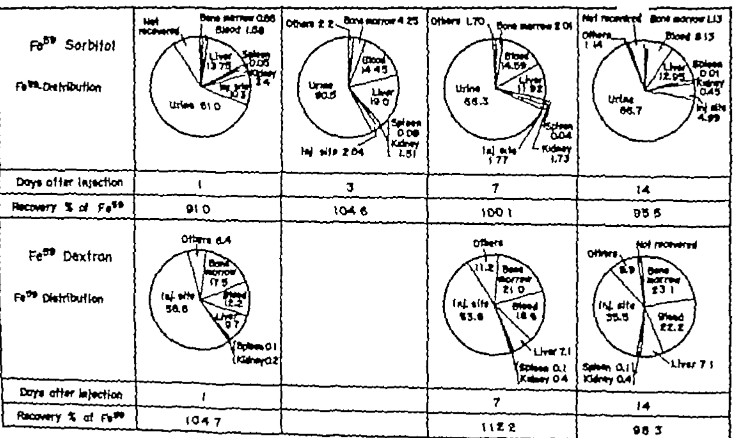


Fig. 1 Radio-iron distribution in various organs of rabbit after single intramuscular injection of Fe^{59}-sorbitol or Fe^{59}-dextran.

Fe^{59}-Sorbitol Fe^{59}-Distribution	Others 0.48, Bone marrow 3.22, Blood 2.08, Not recovered, Liver 12.0, Spleen 0.07, Kidney 2.3, Urine 55.5	Not recovered, Bone marrow 1.78, Blood 3.92, Others 0.18, Liver 17.6, Spleen 0.06, Kidney 1.73, Urine 72.4	Not recovered, Bone marrow 0.75, Blood 0.84, Liver 9.05, Spleen 0.02, Kidney 0.63, Urine 70.6	Not recovered & others, Bone marrow 3.23, Blood 15.95, Liver 5.16, Spleen 0.09, Kidney 1.79, Urine 70.0
Days after injection	1	3	7	14
Recovery % of Fe^{59}	75.65	97.5	90.9	96.2
Fe^{59} Dextran Fe^{59} Distribution	Not recovered, Others 4.0, Blood 7.4, Bone marrow 23.6, Liver 63.0, Kidney 0.7, Spleen 0.6		Kidney 0.7, Spleen 0.6, Others 6.0, Bone marrow 27.7, Liver 48.7, Blood 19.7	Kidney 0.7, Spleen 0.5, Others 3.9, Not recovered, Bone marrow 33.4, Liver 36.3, Blood 18.4
Days after injection	1		7	14
Recovery % of Fe^{59}	91.5		103.6	93.2

Fig. 2. Radio-iron distribution in various organs of rabbit after single intravenous injection of Fe^{59}-sorbitol or Fe^{59}-dextran.

organs. However the lymph node radioactivity fell more rapidly in the case of iron sorbitol

(b) Distribution of Radio-Iron after a Single Intravenous Injection of ⁵⁹Fe-Sorbitol

The results are shown in Fig 2 and Table I It is apparent that after intravenous injection of iron sorbitol the ⁵⁹Fe distribution patterns were similar to those following intramuscular injection, except that slightly more radio-iron was excreted in the urine. A higher plasma radio-iron concentration after intravenous injection may increase the urinary excretion of radio-iron.

Intravenous injection of iron dextran was followed by little or no excretion in the urine and a greater iron distribution to the bone marrow than was the case after intravenous injection of iron sorbitol. The radio-iron concentration in the blood 2 weeks after injection of iron sorbitol and iron dextran was 16 and 18%, respectively

(2) Clinical Results

(a) Utilization and Urinary Excretion of Radio-Iron in Patients with Iron Deficiency Anaemia Following a Single Intramuscular Injection of ⁵⁹Fe-Sorbitol

In all cases the plasma radioactivity reached its peaks of 15 to 25% 3 to 6 h after the injection and then gradually decreased to 2 to 3% or less of the injected radio-iron 12 to 24 h after injection (Fig 3) Radio-iron was taken up by the erythrocytes as early as 12 to 24 h after the injection of iron sorbitol, and it increased more rapidly than in the case of iron dextran. About 2 weeks after injection the utilization rate for iron sorbitol ranged from 54 to 65% and was higher than that for iron dextran. Urinary excretion of ⁵⁹Fe was observed within 3 h of injection. It is apparent from Fig 2 that both plasma content and urinary excretion of radio-iron reached their maximum 3 to 6 h after injection, and then gradually decreased, the excretion, however continued over a period of 48 to 72 h.

(b) Clinical Effects of Iron Sorbitol on Iron Deficiency Anaemia

Increase in haemoglobin. Twelve patients with iron deficiency anaemia received iron sorbitol intramuscularly In all the cases the haemoglobin level rose immediately after initiation of the treatment (Table II Fig 4 and 5) In 7 patients who received 400 mg iron

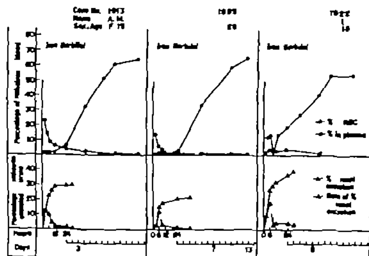


Fig. 3. Plasma iron disappearance, percentage of red-cell iron utilization and urinary excretion of iron in cases of iron deficiency anaemia after single intramuscular injection of Fe^{59}-labelled iron sorbitol (iron 90 mg)

Table II
Haematologic data for the patients treated with iron sorbitol.

No.	Case Name	Sex/age	Prior to treatment Hb g/dl	RBC/Ht	SI γ/dl	UIBC γ/dl	After treatment Hb g/dl	RBC/Ht	SI γ/dl	UIBC γ/dl
61–138	M. A.	F/44	7.35	25	44	475	15.0	40	103	177
62 2	M. S.	F/23	7.6	24	41		14.27	41	103	177
62 3	M. M.	F/42	7 78	26	35	430	14.1	45	106	273
62 6	O. K.	F/20	9.05	28	23		14 8	42	103	301
62 20	U M.	F/21	7.0	30	35	410	13.6	43	106	311
62 64	S. K.	M/33	11.2	38	32	259	15.35		62	356
62 65	S. Y	F/20	11.2	33			16.0	49	68	363
1868	T K.	F/36	6.0	2.85	40	392	13.7	4.95	221	137
1895	K. H.	F/26	10.35	3.30	47	396	15.0	5 48	123	168
1912*	U F	F/44	5.7	2.25	58	386				
1913	A. M.	F/15	8.75	2.85	38	473	14.0	4 75	160	242
1922	A. L.	M/18	9.5	3.82	26	498	15.4	4.90	78	256

× 10^6 mm^3
Discontinuation of the treatment due to allergic eruption

weekly the mean daily increase ranged from 0 09 to 0.34 g/dl during the first 2 to 3 weeks in 5 who received 700 mg iron weekly the corresponding increase varied between 0 10 and 0 4? g/dl. The mean daily increase was 0.20±0 08 g/dl for the former group and 0.21±0 16 for the latter (95% confidence limit) There is no significant difference between these values (P <0 05) The mean daily

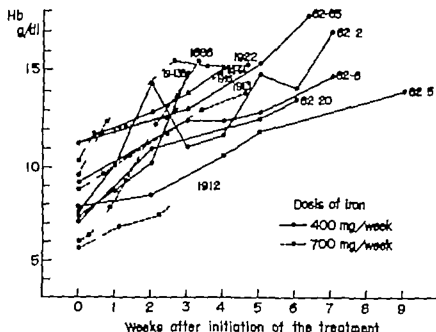


Fig 4 Change in haemoglobin concentration after initiation of the treatment with iron sorbitol.

increase until the concentration returned to normal was 0.18 ± 0.08 and 0.24 ± 0.17 g/dl, respectively the difference is not significant. Nor was any significant difference found between these results and those previously reported for intramuscular injection of iron dextran or peroral iron medication (Table III)

Excretion with the urine The 24-hour urinary excretion of iron was determined in 3 patients daily or at irregular intervals during the treatment. The results are recorded in Fig 5 Urinary excretion of iron varied from day to day ranging from 12 to 97 mg It is noteworthy that massive iron excretion exceeding 90 mg was occasionally encountered.

Changes in serum iron level and UIBC after treatment. In 11 patients the serum iron level and unsaturated iron binding capacity of serum were determined several days after completion of treatment. The results are shown in Table II and Fig 5 In some cases, the serum iron levels were still below normal and UIBC remained higher than normal this points to iron deficiency even though the haemoglobin concentration was normalized. The fact that the calculated iron dose failed to cover the deficiency seems to have been due to excess iron excretion with the urine.

Table III

Mean daily increase in haemoglobin concentration in patients with iron deficiency anaemia following parenteral or oral treatment with various preparations.

		Dose of iron (mg)	No. of cases failed/whole	Daily increase in Hb concentration (g/dl) first 2–3 weeks	whole course
Iron sorbitol	parenteral	400 week	7/7	0.20±0.08	0.18±0.08
		700	5/4	0.21±0.16	0.24±0.17
Iron dextran		350–400	5/4	0.27±0.18	0.17±0.13
		600–700	15/12	0.23±0.09	0.21±0.06
Ferrous fumarate	peroral	126 day	11/	0.26±0.12	
		189	6/4	0.25±0.11	0.15±0.04
Ferroglycine sulphate		138	11/	0.22±0.08	
		207	5/	0.21±0.12	
Ferrous succinate		160	6/5	0.31±0.14	0.20±0.12

Confidence limits of the mean concentration ($P = 0.05$)

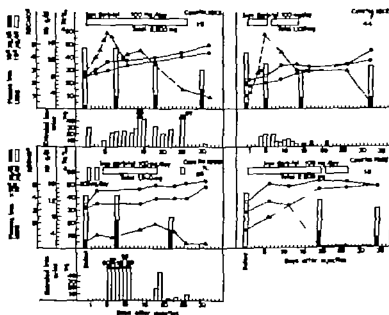


Fig. 5. Effect on anaemia, changes in serum iron and UIBC levels and urinary excretion of iron after treatment with iron sorbitol.

Side effects. All the patients receiving intramuscular injections of iron sorbitol complained of pain at the site of injection, especially after the first injection. A few reported difficulty in walking due to

pain but neither neurologic nor inflammatory signs were noted. There was occasionally slight pigmentation of the skin. Mild nausea of 30 to 60 minute duration sometimes followed a daily dose of 200 mg iron, but not even these patients showed hypotension. No such signs were noted in cases of a daily dose of 100 mg iron. Allergic eruption was observed in one patient after the eleventh injection of iron sorbitol, but it disappeared spontaneously a few days after the injections had been discontinued. A positive skin reaction to iron sorbitol was obtained in this case. No other noteworthy side effects were observed.

Comment

In recent years many studies have extended our knowledge of the metabolic behaviour of colloidal iron. However a number of problems remain to be solved, since the nature of this colloid may vary according to the type of preparation.

In this investigation the distribution of radio-iron, the process of the utilization and its excretion were examined after injection with ^{59}Fe-sorbitol. The study was performed on normal animals and on cases of iron deficiency anaemia. The results were compared with those reported for iron dextran. In normal rabbits the two substances showed significant differences in the iron clearance from the site of injection and in the urinary excretion of iron. Only 10% of the radioactivity remained at the injection site 24 h after injection of ^{59}Fe-sorbitol. The corresponding figure for iron dextran was about 60%.

The fact that after intramuscular injection of ^{59}Fe-sorbitol in rabbits the maximum radioactivity was attained more rapidly in lymph nodes near the site of injection than elsewhere suggests that iron sorbitol, like iron dextran is transported via the lymphatics.

As the lymph node radioactivity 24 h after injection was appreciably lower for ^{59}Fe-sorbitol than for ^{59}Fe-dextran, the former agent may well have been transported more rapidly via the lymphatics. The fact that ^{59}Fe is excreted in the urine after injection of iron sorbitol but not of iron dextran, and that the radio-iron distribution patterns are similar for intravenous and intramuscular injection of iron sorbitol suggests that this may be transported unchanged from the site of injection into the blood stream. ANDERSSON (1) reported that after a single injection of iron sorbitol equivalent to 100 mg iron in patients with iron deficiency anaemia about 30% of the

iron was excreted via the kidneys. This is confirmed by the present results and those of many other investigators (19 24) The utilization of ^{59}Fe at 13 days was about 60% for a single injection of ^{59}Fe sorbitol.

Intramuscular injection of iron sorbitol in 12 patients with iron deficiency anaemia produced a satisfactory rise in the haemoglobin level. As previously reported (12) in oral therapy for essential iron deficiency anaemia the rate at which haemoglobin was formed rose in proportion to the iron dose up to about 140 mg daily but further increase produced no change. The maximum daily rise in haemoglobin was 0.21 g/dl.

For intramuscular injection of iron dextran in doses of 350 to 400 mg per week the daily increase in haemoglobin attained a maximum of 0.21 g/dl (12) The corresponding value for iron sorbitol was 0.2 g/dl. In 3 cases of iron deficiency anaemia, the iron excretion via the kidneys varied from day to day The 24-hour renal iron output exceeding 50% of the injected dose frequently encountered indicates an inter-individual variation in the total urinary excretion of iron during the treatment. Thus, in some cases 30% in excess of the calculated amount did not cover the urinary excretion of iron. In consequence low serum iron and high UIBC levels may persist after completion of the treatment, even though the haemoglobin level returns to normal. For this reason it is advisable to monitor the urinary excretion of iron throughout the treatment of iron deficiency anaemia, and to add the excreted amount to the theoretical required amount.

In the determination of the urinary iron content during treatment with iron sorbitol differences in the colour of the urine seemed to be roughly proportional to the iron content. If this is actually the case colorimetric determination should be carried out as early as possible, since the colour alters quite rapidly (17)

Summary

The distribution of radio-iron was studied after single intramuscular injection of ^{59}Fe-sorbitol in the normal rabbit. The amount of residual radio-iron was small, and more than 60% was excreted with the urine. There was evidence of the transport of part of the iron through the lymphatics. In 3 iron-deficient patients, 30% of the radio-iron was excreted with the urine and 60% was utilized for haemoglobin synthesis after single injection of ^{59}Fe-sorbitol. Excellent results in the treatment of iron deficiency anaemia were obtained with doses of 400 mg of iron weekly The amount of the iron excreted varied it occasionally reached 90 mg daily

Zusammenfassung

Bei normalen Kaninchen wurde die Verteilung von radioaktivem Eisen nach einmaliger intramuskulärer Injektion von ^{59}Fe-Sorbitol untersucht. Die Menge des verbleibenden radioaktiven Eisens war gering, und mehr als 60% wurden im Urin ausgeschieden. Ein Teil des Eisens wurde durch die Lymphwege transportiert. Bei 3 Patienten mit Eisenmangel wurden nach einer einmaligen Injektion von ^{59}F -Sorbitol 30% des radioaktiven Eisens im Urin ausgeschieden und 60% wurden bei der Hämoglobinsynthese erwertet. Bei der Behandlung der Eisenmangelanämie wurden mit einer Dosis von 400 mg Eisen pro Woche ausgezeichnete Resultate erzielt. Die Menge des ausgeschiedenen Eisens variierte, sie erreichte gelegentlich 90 mg täglich.

Résumé

La répartition de fer radio-actif été étudiée chez des lapins normaux après une injection intramusculaire unique de Fe^{59}-sorbitol. La quantité de fer radio-actif retenu par l'organisme était faible, plus de 60% étant excrétés dans les urines. Une partie du fer était transportée par les voies lymphatiques. Chez 3 malades atteints d'un manque de fer 30% du fer radio-actif furent excrétés dans les urines après une injection unique de Fe^{59}-sorbitol, et 60% furent incorporés dans la synthèse de l'hémoglobine. Les résultats obtenus à l'aide d'une dose de 400 mg de fer par semaine dans le traitement d'anémies par manque de fer furent excellents. La quantité de fer excrété varie, n tteignant qu occasionnellement 90 mg

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Study on Nine Families with Haemoglobin Lepore in Campania

Hb Lepore Trait Heterozygosity for Hb Lepore and β-Thalassaemia, Homozygosity for Hb Lepore

N Quattrin P Bianchi R. Cimino, L. De Rosa, E. Dini and V Ventruto

In 1958 during a study on a M Cooley's family of italian origin, Gerald and Diamond (1) have discovered a new slow haemoglobin variant associated with thalassaemia like findings. Such anomaly was called Hb Lepore $_{Boston}$ Other similar cases have been successively observed in Italian (2 3 4) Greek (5 6) Papuan (Hb Lepore $_{Hollandia}$) (7) Turkish Cypriot (Hb Lepore $_{Cyprus}$) (8) and Afro-American (Hb Lepore $_{Th. Baltimore}$) (9) subjects. It has to be noticed that in the Greek individuals this abnormal haemoglobin was firstly designated as Hb Pylos but is presently considered as one of the Hb Lepore variants.

There are about sixty cases so far known that could be divided in four states (a) heterozygous carriers (b) double heterozygosity for Hb Lepore and beta thalassaemia (1 3 4 5 8) (c) double heterozygosity for Hb Lepore and Hb S (2 6) or Hb C (9) (d) homozygous subjects (5 7) On the base of the above stated observations it results that while carriers of Hb Lepore alone are sound, the double heterozygous and the homozygous individuals are affected by an anaemia similar to the thalassaemia major

Very interesting are Baglioni's biochemical studies (10, 11) showing that haemoglobins Lepore $_{Boston}$, Lepore $_{Hollandia}$ and Pylos consist of a fusion product of both delta-chains and beta chains, the N terminal end and part of the delta chain being joined

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to the C-terminal end and a portion of the beta-chain. In this way a new hybrid globin chain forms, which is synthetised with a very slow rate. On the other hand the beta and delta chains consequent deficiency explains the risen amount of Hb F and the tendency to lower levels of Hb A_2, noticed in the heterozygous individuals with Hb Lepore variants. This same being furthermore the reason why in the homozygous state for Hb Lepore the haemoglobins A and A_2 were ordinarily absent.

Methods and Material

During the past five years our staff has been investigating upon the presence of thalassaemia and other haemoglobinopathies in over 14,000 people in Campania (12, 13). The general results of this study are reported in Table I and II. In this work the cases with Hb Lepore and related syndromes, are reported. The following methods were used.

The hysates of haemoglobin were prepared by standard technique (18). Haemoglobin solutions were analysed by the following methods:

(a) starch gel electrophoresis in borate buffer pH 8.6 (14)
(b) agar gel electrophoresis at pH 6.2 (15)
(c) starch block electrophoresis in borate buffer pH 8.6 (16)
(d) paper electrophoresis in TRIS buffer pH 9.2 (17)
(e) paper electrophoresis in phosphate buffer pH 6.5 (18);
(f) starch gel electrophoresis in the discontinuous buffer system (19)
(g) solubility test (20)
(h) alkali resistance test (21)
(i) column chromatography on amberlite resin CG-50 type II (22)
(k) fingerprinting of the haemoglobin (23) and of the carboxymethylated globin, using modified chromatographic solvent (24)

Results

In Table III are summarized some data concerning nine unrelated families with Hb Lepore. Out of 33 investigated subjects 19 were found to be carriers of the anomaly and two others affected respectively by homozygosity and double heterozygosity for Hb Lepore and beta thalassaemia. These individuals were of both sexes and all ages. The haemoglobin anomaly has been found in three (Avellino, Caserta, Napoli) out of the five provinces of Campania, but most of the cases (11) and families (6) belong to the province of Caserta. All the sound heterozygous carriers showed a mild thalassaemia like blood picture and decreased osmotic fragility. The levels of the haemoglobins Lepore, A_2 and F found in these subjects are reported in Table IV resulting of the comparison of literature data (25) and ours. Considering these table it appears a good correspondence of our data with those of the other Hb Lepore

Table I
Materials and general results.

Investigated subjects	14524	—
Subjects with thalassemic traits or diseases	916	(6.3%)
Subjects with other Hb anomalies	96	(0.6%)
Thalassemic subjects		91
Haemoglobinopatic subjects		9

Table II
96 Cases of haemoglobinopathy

Carriers of Hb S	37
Carriers of Hb D	12
Carriers of Hb C	4
Carriers of Hb Caserta	3
Carriers of Hb Mexico	1
Carriers of Hb Lepore	19
Carriers of Hereditary Persistence of Hb F	1
SILVESTRONI-BIANCO Disease	14
Drepanocytic Anaemia	1
Hb Bart in Homozygous alpha-Th	1
Hereditary Persistence of Hb F + beta-Th	1
Hb Lepore + beta-Th	1
Homozygosity for Hb Lepore	1

Table III
Nine families with Hb Lepore

No.	Name	Province	No. of tested subjects	Heterozygous	Homozygous	Heterozygosity fo Hb Lepore and beta-thalassemia
1	Cent.	Caserta	1	1	—	—
2	Bell.	Caserta	3	2	—	—
3	Giang	Caserta	5	3	—	—
4	Par	Caserta	1	1	—	—
5	Pun.	Napoli	2	2	—	—
6	Na.-Bo.	Caserta	4	2	1	—
7	Ri. Ra.	Avellino	5	2	—	1
8	Mar	Napoli	5	3	—	—
9	Rus.	Caserta	7	3	—	—
		Total	33	19	1	1

variants In our carriers however it results on average quite risen the amount of anomalous haemoglobin, while the Hb F level was a little lower

Discussion

The Hb Lepore is a not rare anomaly in Campania, its frequency being second, according to our experience, just to Hb S and thalassaemia. What seems to us quite remarkable (vide Adden dum) The first cases in Italy were described by SILVESTRONI, BIANCO and BRANCATI (2, 3 4) who observed also subjects with double heterozygosity (Hb Lepore + beta thalassaemia and Hb Lepore + Hb S) It has to be noticed that most of these subjects too were from the province of Caserta. Our researches fully confirm the already known haematological patterns of the Hb Lepore carriers and precisely the thalassaemia like picture of the erythrocytes, the HbF growth and the tendency to lower Hb A_2 levels.

A short comment deserve our two observations, concerning respectively the cases with double heterozygosity and homozygosity

Heterozygosity for Hb Lepore and beta thalassaemia (Table III Ri Ra Family) It is about a 12 years old boy from the province of Avellino, whose mother and father resulted to be carriers of beta thalassaemia and Hb Lepore respectively a sister of the patient is a Hb Lepore carrier too, while the other one resulted to be normal. The boy appears regularly grown but presents anaemia, icterus and hepato-splenomegaly while Cooley's facies is absent. His parents say that such a symptomatology began when he was 4 or 5 years old. In spite of the illness the boy has always attended school *profitably*

The haemoglobin patterns gave the following data Hb Lepore 12 % Hb F 30 % Hb A_2 1.2 % the remainder being Hb A. In the father and sister both heterozygous carriers, the Hb Lepore

Table IV

Comparison of some quantitative data of haemoglobins in various heterozygous Lepore variants.

	Hb Lepore ([illegible])	*Hb Lepore* ([illegible])	*Hb Pylos*	*Hb Lepore* (Cyprus)	*Our cases*
Number of cases	8	11	6	6	19
Percent Hb F	1.5—18 (mean 4.7	2 7—8.2 (mean 5.0)	3 8—14 (mean 4.7	0—7 mean 4.6	0 7—8 (mean 3.2)
Percent Hb A_2	1.8—2.7 (mean 2.2)	1 2—2 8 (mean 2.1)	1 3—2 6 (mean 2 0)	(low	1 —2 4 (mean 1 9)
Percent Lepore variant	7—12 (mean 10 6)	10—13 (mean 10.8)	6—12 (mean 8.2)	5—13 mean 8.2	8—20 (mean 15

Table V
Haematological data from the members of the family Na. Bo.

	Age (years)	Hb (g%)	R. B. C. (million/mm³)	C.I.	M.C.V.	Reticulocytes ( )	Hb type	Hb Lepore ( )	Hb A_2 ( )	Hb F ( )	Solubility test (Itano)	Osmotic resistance (SIMMEL, 0.4)	Anisocytosis	Poikilocytosis	Target cells	Serum iron (μg)	Bilirubinaemia in d. (mg %)
I 1	56	16	6.8	0.72	76	0.7	A+ Lepore	18	1.7	5.8	neg.	14	++	++	++	145	1.3
I 2	25	13	6.2	0.63	68	1.3	A+ Lepore	15	2.1	8	neg.	19	++	++	++	138	1
II 3	3½	9	3.6	0.74	72	2.4	F+ Lepore	30	ba.	70	neg.	63	+++	+++	+++	150	1.2
II, 4	2	13	6.2	0.72	86	1.2	A	abs.	2.4	1.2	neg.	1.2	ba.	abs.	abs.	92	0.7

Propositus

level was 11 % and 17 % respectively These results confirm in the state of double heterozygosity for Hb Lepore and beta thalassaemia, the amount of Hb Lepore is the same as that found in persons heterozygous for Hb Lepore alone, or lower Since the age of 7 years the patient has been periodically treated with numerous blood transfusions. Lately we suggested the splenectomy but the parents were opposed to it.

Homozygosity for Hb Lepore (Table III Na Bo Family) The propositus is a 4 years old child from the Caserta province, whose parents are both carriers for Hb Lepore trait, while his little sister is normal (Fig 1) The child was born and grew regularly The family doctor noticed just a tendency to icteric pallor and a light splenomegaly The patient does not present either the Cooley s facies or bone changes at the radiological examination. The haematological findings of the four members of this family are summarized in Table V Fig 2 shows the electrophoretic patterns of the haemoglobins. These researches demonstrate clearly that the blood pigment of the ill child consists exclusively of Hb F (70) and Hb Lepore (30%) On the contrary Hb A and Hb A_2 are completely absent. These same features have been found in the homozygosity for Hb Pylos (5) while in two homologous cases described by NEEB *et al* (7) traces of Hb A_2 were demonstrated by carboxymethyl

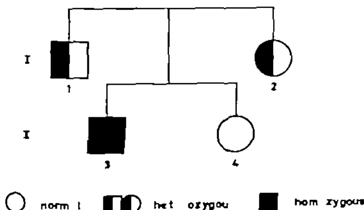


Fig 1 Hb Lepore Fam. Na-Bo

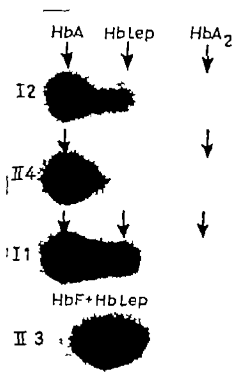


Fig. 2. Electrophoretic patterns of the family Na Bo. (Starch gel electrophoresis) I II 3 homozygosity for Hb lepore) Hb A and Hb A_2 are absent.

cellulose chromatography only. This particular behaviour being in full accordance with BAGLIONI's (10, 11) above mentioned studies.

Our case is however quite interesting not only on account of the great rarity of Hb Lepore homozygosity but also for it seems to be clinically different from the other three cases, up to now described. Unlike the observations of NEEB *et al.* (7) and FERRAS *et al.* (5) our child does not present so far any Cooley's symptomatology.

The results of the study on our two cases of Hb Lepore anaemia lead us to the conclusion that the different seriousness of clinical findings, noticed by the various authors, can be led black to a molecular not quite clear yet though, difference of the Hb Lepore single variants. Thus the homozygous and respectively double het erozygous states for this haemoglobinopathy would cause therefore phenotypes with different clinical findings and courses.

Summary

The authors report the results of their researches about the presence of Hb Lepore in Campania. Nine families, comprehending 19 heterozygous, 1 homozygous and 1 double heterozygous (Hb Lepore + beta thalassaemia) subjects, have been studied. After the thalassaemia and sicklaemia the Hb Lepore appears to be the most frequent haemoglobin anomaly in this region. The authors confirm the thalassaemia-like picture of the erythrocytes, the Hb F growth and the tendecy to lower Hb A_2 levels in the Hb Lepore carriers. A case of homozygosity for Hb Lepore is discussed which is the fourth so far described. The blood pigment of this 4 years old patient consisted exclusively of Hb Lepore (30%) and Hb F (70%). In opposition to the other 3 cases already known this child did not present the Cooley's features. The authors believe therefore the Hb Lepore variants can differ in one way from one another

Zusammenfassung

Es werden Untersuchungen über das Vorkommen von Hb Lepore in der Campania mitgeteilt. Sie betreffen 9 Familien mit 19 heterozygoten Individuen, einem homozygoten und einem doppelt heterozygoten (Hb Lepore + β-Thalassämie) Individuum. Nach der Thalassämie und der Sichelzellkrankheit dürfte Hb Lepore die häufigste Hämoglobinanomalie in dieser Gegend darstellen. Das Thalassämie-ähnliche rote Blutbild, die Vermehrung von Hb F und die Tendenz zu niedrigen Werten von Hb A_2 bei den Trägern von Hb Lepore werden bestätigt. Ein homozygoter Träger von Hb Lepore wird beschrieben, bei dem es sich um den vierten bisher mitgeteilten Fall handelt. Der Blutfarbstoff dieses 4 Jahre alten Patienten bestand ausschließlich aus Hb Lepore (30%) und Hb F (70%). Im Gegensatz zu den anderen 3 Fällen wies dieses Kind keine Eigenschaften der Cooley-Anämie auf. Die Autoren vertreten die Meinung, dass zwischen den Varianten des Hb Lepore Unterschiede bestehen

Résumé

Les résultats de recherches concernant la presence d'Hb-Lepore en Campanie sont rapportés. Neuf familles comprenant 19 personnes heterozygotes, une homozygote et une doublement hétérozygote (Hb-Lepore et beta-thalassémie) ont été étudiées. L'Hb-Lepore semble être l'anomalie de l'hémoglobine la plus fréquente dans cette region après la thalassémie et la drépanocytose. La ressemblance des érythrocytes à ceux de la thalassémie, l'augmentation du taux d'HbF et la diminution de celui d'HbA_2 chez les porteurs d'Hb-Lepore sont confirmées. Le cas d'un porteur homozygot d'Hb-Lepore est décrit, le quatrième de ce genre rapporté jusqu'à maintenant. L'hémochrome de ce malade âgé de 4 ans était constitué uniquement d'Hb-Lepore (30%) et d'HbF (70%). A l'encontre des trois autres cas, cet enfan ne presentait aucune des particularités de la thalassémie. Les auteurs en déduisent qu'il existe des differences entre les variantes de l'Hb-Lepore.

Addendum. While this work was lying in typography the authors had the opportunity to study 14 more cases of Lepore trait and 1 other case of double heterozygosity for Hb Lepore and beta-thalassaemia. This new experience brings up to 36 our Hb Lepore observations.

All these cases, belonging to 20 different families from the provinces of Naples, Caserta and Avellino increase even more the epidemiological and nosological importance of this anomaly in Campania

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Hereditary Methemoglobinemic Cyanosis Due to Diaphorase* Deficiency in Three Successive Generations

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Acquired methemoglobinemia may occur fairly frequently as a result of the administration of oxidative drugs or compounds (1). However, hereditary methemoglobinemic cyanosis is a rare condition. The persistent slate-gray cyanosis of patients with this entity is due to an excess of methemoglobin which comprises 5 to 60% of the total hemoglobin. Although the disease is usually well tolerated, if the concentration of this ferrihemoglobin is high, easy fatigability, palpitation, severe headaches and dyspnea on exertion may be present.

It is now clearly established that methemoglobinemia of the non-acquired type is a molecular disease. However, the precise pattern of inheritance is not fully elucidated. Sporadic, non acquired types of methemoglobinemia cases have been simply termed 'congenital' or 'idiopathic'. When the disease has been found in one or more siblings, the terms 'familial congenital', 'familial idiopathic methemoglobinemia' or 'hereditary methemoglobinemic cyanosis' have been used (2). In cases of dominant inheritance of the disease, patients usually have an abnormal hemoglobin, Hemoglobin M (3, 4). Congenital methemoglobinemias due to methemoglobin diaphorase deficiency are recessively inherited (3, 5–9).

We have studied a family in which six members in three generations have had methemoglobinemia with methemoglobin diaphorase deficiency. Since this fairly rare condition has not been reported from Turkey and the recessive inheritance is very unlikely in this pedigree, we thought it worthwhile to publish our findings.

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* Methemoglobin diaphorase and diaphorase refer to DPNH dependent methemoglobin reductase.

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Case Report

A. E. (T T F 65/7292) is a 39 years old Turkish female who was referred to the Hematology Clinic from the Cardiology Department because of generalized cyanosis with the possible diagnosis of methemoglobinemia. She had been noted to be cyanotic since birth. She further stated that when she lost weight her cyanosis worsened. Her complaint was dyspnea on exertion for year duration, and intermittent headaches especially when she was nervous. During the past year she was examined in tw other hospitals and cardiac catheterization was advised. She refused it and was treated with different medications which did not cause any improvement.

Physical examination revealed well-developed, well-nourished white female with marked cyanosis which was pronounced in her lips, external ears and nail beds The palpebral conjunctivae were of brown hue. There was no clubbing of the fingers The lungs were clear to percussion and auscultation and the heart sounds were of good quality with regular rhythm. No murmurs were detected and the heart size was within normal limits. The remainder of the examination was unremarkable. Her blood pressure was 110/70 and her pulse rate 80/min.

The hemoglobin was 15.4 g/100 ml of blood the WBC, 6500/mm³ with normal differential count. The sedimentation rate was 24 mm/h. The urinanalysis, NPN, FBS, Na Cl- CO_2, total protein, cholesterol, ECG and chest X-ray were all within normal limits.

As it is seen from her family tree (Fig 1) her mother brother tw sisters and one of her children has cyanosis. By history one of her aunts had the disease but was not available for study The propositus and her family liv in Ankara. Her parents and siblings liv in village near Ankara. No one else in the village has cyanosis.

Methods

The methemoglobin and fetal hemoglobin (HbF) concentrations were determined by the method of Evelyn and Malloy (10) and Singer (11) respectively The erythro-

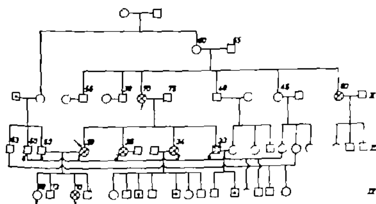


Fig 1 The pedigree of the methemoglobinemic family

□ Male ○ Female ⊗ ⊠ Affected persons ○□ Deceased

(Numbers over the signs indicate person present age or age at death)

Table I
The results of laboratory studies.

	Ages	Hc	Hb g	MetHb	G-6PD μm^*	RBC glutathione level before and after stability test	Blood group
E. (III.1) propositus	39	44	15.4	19	23	66/58.4	A+
D. (II.1)	0	40	12.83	35.5	18.5	108.7/94	O+
D (II.2)	73		14 17	0.5			A+
G. (III.2)	25	48	16.17	24.5	16.9	98/81	A−
A. (III.3)	34	48	16.42	20.5	17	102.5/78	A+
D (III 4)	23		14.31	11			A−
E. (III.5)	50	46	15.4	0	13.9	93/71	O+
A. E. (III.6)	53	47	16.4	0	22.5	84/50	A+
E. (IV 1)	18	45	15	0	13.7	101/66	A+
. (IV.2)	13		11.83	0.25			O+
E. (IV.3)	10	45	13	30	18.5	66/58	A−
E. (IV 4)	7	41	11 75	0.55	20	92.7/73.2	O+

Indicates family member with methemoglobinemia.
normal over 10 μm

cyte reduced glutathione glutathione stability glucose-6-phosphate dehydrogenase and DPNH -dependent methemoglobin diaphorase activity determinations were performed by the methods of BEUTLER (L.), GRUNERT and PHILLIPS as modified by BEUTLER (13), SCOTT with the modification of ROSE (14) and NAKAMARA-OZAND (15), successively. The Heinz body preparations were done as described by DEAN *et al.* (16) The starch gel electrophoresis was performed according to SMITHIES (17) and the agar gel according to ROBINSON *et al* 18 The hemoglobin level was determined as cyanmethemoglobin. For the spectroscopic studies, Beckman's D U spectrophotometer was used. The benzoquinone acetic acid in the urine was tested according to FISHMAN 19). A possible inhibitor effective on DPNH-dependent methemoglobin diaphorase was checked by the assay of this enzyme 1 1 mixture normal and the propositus hemolysates.

Results

The hemoglobin, Hct* methemoglobin, G-6PD* GSH* glutathione stability and blood group results of the propositus and the examined members of this family are given on Table I

Examination of the propositus blood revealed a methemoglobin concentration of 19 of the total hemoglobin She was given methylene blue (3 mg/kg) by mouth and the methemoglobin level the next day was found to be 0.7 %. The hemoglobin electrophoresis on starch gel at pH 7 (phosphate buffer 0 006 M) is shown on Fig 2. The control and the patient s oxy meth- and cyanmethemoglobin

DPNH reduced diphosphopyridine nucleotide *Hct* hematocrit. *G-6PD* glucose six-phosphate dehydrogenase. *GSH* reduced glutathione.

levels did not reveal any difference. Neither the starch gel electrophoresis at pH 8 6 (Tris-citric acid) nor the agar gel at pH 6.45 (citrate-phosphate) showed any abnormal hemoglobin component in the patient s hemolyzate.

The absorption spectra were obtained with the dilution of the propositus blood 1:20 at pH 7.3 (phosphate buffer 0 1 M) By the addition of potassium ferricyanide, the peak around 630 nm disappeared and that time there was not any spectral difference between the control cyanmethemoglobin A and the patient s specimen These findings confirm that the patient has no demonstrable hemoglobin abnormality The above studies were performed with the blood of other members of this family and no abnormal hemoglobin could be demonstrated.

In the urine of these cases, benzoquinone acetic acid was not found and the serum vitamin C levels were normal, as were the erythrocyte GSH and glutathione stability No methemoglobin diaphorase activity was found in the red cells of the propositus and



Fig 2 Hemoglobin electrophoresis on starch gel at pH phosphate buffer 0 0? M The control and the patient oxyhemoglobin (upper two methemoglobin middle two) and cyanmethemoglobin (lower two did not show any difference Benzidine stain

her other family members with methemoglobinemia, and no inhibitor was shown that could be effective on this enzyme activity.

The HbF concentrations and Heinz bodies in the erythrocytes of these patients were not significantly different than in the control cases.

Discussion

There are several kinds of the non acquired type of methemoglobinemias. Methemoglobinemia due to the presence of the abnormal hemoglobin M, like other hemoglobinopathies, is inherited as a dominant characteristic (3, 4).

The great majority of congenital methemoglobinemia cases reported in the literature were due to methemoglobin diaphorase deficiency which appears to be transmitted recessively as stated above (3, 5–9). Although CODOUNIS (20) reported four genealogical trees with dominant inheritance of hereditary methemoglobinemic cyanosis, in these cases the deficiency of diaphorase activity was proved only recently (21).

TOWNES *et al.* (22, 23) reported a male patient with hereditary methemoglobinemia whose family history indicated dominant inheritance. The only abnormality which could be shown in this case was d[illegible]ased red blood cell GSH and oxidized glutathione. However [illegible]mportance of GSH for the reduction of methemoglobin rem[illegible] uncertain and the erythrocyte GSH level in hereditary me[illegible]globinemic cyanosis cases with diaphorase deficie[illegible] ha[illegible] been found to be normal (6).

Another type of methemoglobinemia possibly due to a metabolic d[illegible] with the excretion of benzoquinone acetic acid in the urine [illegible]ith hypovitaminosis C was reported in a case by FISHBERG (19).

TPNH[illegible] Methemoglobin reductase is another enzyme which could be [illegible]ffective on methemoglobin reduction. However methemoglobinemia due to the deficiency of this enzyme has not been reported, and its activity has been assayed in at least two cases of congenital methemoglobinemia with diaphorase deficiency and was found to be normal (24).

All of our patients had had their cyanosis since birth and their methemoglobin values were fairly high. Our studies indicated that

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TPNH Reduced, triphosphopyridine nucleotide

methemoglobinemia in our subjects was not due to the presence of the abnormal hemoglobin M. In our patients the erythrocyte GSH level was not low and we found neither the excretion of benzoquinone acetic acid in the urine nor hypovitaminosis C. Moreover the methemoglobin diaphorase activity was almost absent in the red cells of all of our cases. We could not find an inhibitor effective on methemoglobin diaphorase. All of these patients responded to methylene blue (2–3 mg/kg) treatment by mouth.

TPNH which is generated by G-6PD activity is essential for TPNH dependent methemoglobin reductase activity Methemoglobin diaphorase and G-6PD deficiency together is reported (25 26) Therefore we assayed the red cell G-6PD activity and found it to be normal in our cases. Although low serum vitamin C levels, which may alternate with the season, have been reported in congenital methemoglobinemia (2, 6 20) normal vitamin C levels were found in our patients.

The inheritance of the disease in this family (Fig 1) is very unlikely to be recessive. If it were recessive it would be impossible to observe any affected offspring when one parent only was effected (II.1 and IV.3) unless the other parent (II.2 and III.5) was a heterozygote. In the recessive form, although heterozygotes are asymptomatic, they could be detected by methemoglobin diaphorase assays (5) The diaphorase activity was measured and found normal in the erythrocytes of the propositus father (II.2) and her husband (III.5) Also the inheritance of this disease does not appear to be sex-linked as both sexes are affected in this family If it were a regular dominant inheritance, one of the propositus grandparents (I) would have had methemoglobinemia. If it were an irregular dominant inheritance, one would expect to find a case among the parents of siblings of the propositus grandparents. The maternal side of the propositus grandmother is shown in the figure but more information could not be obtained about the grandfather s family Because *the propositus mother and one aunt* also had the disease, it also cannot be explained with mutation. Since we could not obtain more information about the propositus' grandfather's family the dominant inheritance of the disease could not be rejected in this family

In two cases of congenital familial methemoglobinemia Barcke and Valtes (27) found that the oxygen dissociation curve of their patients became normal following methylene blue ad-

ministration, but not with vitamin C. For this reason and because it is cheaper we have been using methylene blue in the treatment of our patients.

In conclusion, in our cases, the erythrocyte diaphorase activity was zero and *other causes of methemoglobinemia were not present*, and the dominant inheritance of hereditary methemoglobinemic cyanosis due to enzyme deficiency is most likely.

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Summary

Six cases of familial methemoglobinemic cyanosis due to methemoglobin diaphorase deficiency in three generations of one family are reported. The inheritance and the types of congenital methemoglobinemia are briefly discussed. The inheritance of this molecular disease in this family seems to be dominant. No inhibitor effective on methemoglobin diaphorase could be found in the propositus. All the affected members of this family responded to methylene blue treatment.

Zusammenfassung

Es wird über 6 Fälle von familiärer Zyanose infolge Mangels an Methämoglobin-Diaphorase in 3 Generationen einer Familie berichtet. Erbgang und Formen der kongenitalen Methämoglobinämie werden kurz erörtert. Der Erbgang dieser Molekularkrankheit scheint in der vorliegenden Familie dominant zu sein. Es konnte kein Hemmstoff der Methämoglobin-Diaphorase gefunden werden. Alle befallenen Glieder der *Familie sprachen auf Behandlung mit Methylenblau an.*

Résumé

6 cas de cyanose familiale causée par un manque de diaphorase de methémoglobine dans 3 générations d'une famille sont rapportés. Le mode de transmission génétique et les différentes formes de methémoglobinémie sont brièvement discutés. L'hérédité de cette maladie moléculaire semble être dominante dans cette famille. Aucun inhibiteur de la diaphorase de la methémoglobine ne put être trouvé chez le propositus. Tous les membres atteints de cette famille répondirent favorablement au traitement au *bleu de méthylène.*

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Hereditary Methemoglobinemia Due to DPNH Methemoglobin Reductase Deficiency

Report of Family

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Hereditary methemoglobinemia is a very rare condition characterized by cyanosis, a variable amount of hemoglobin in the oxidized form methemoglobin and a compensatory polycythemia. There appear to be two distinct red cell defects causing this condition. The least common is an abnormality of the hemoglobin first demonstrated by Hörlein and Weber (16) and now known as *Hb M* (36) *Hemoglobin M is transmitted as a co-dominant char*acteristic. Individuals with this abnormal hemoglobin have been detected in various parts of the world (1 2, 8, 9 10 11) and some *of these methemoglobins have been found to have different absorp*tion spectra due to different single amino acid subtitutions in the α- or β-polypeptide chain of hemoglobin (1 2 8, 9 10, 11 14 19 22 23 27 30 35 37) The absorption spectra and the electrophoretic behavior of the methemoglobin M variants are characteristically different from those of normal acid methemoglobin. The cyanosis of affected individuals is not altered by the administration of methylene blue or ascorbic acid.

A more common condition is thought to result from a deficiency of an enzyme system called 'methemoglobin reductase In normal human erythrocytes approximately 1% of the total hemoglobin is in the form of methemoglobin. This constant concentration is maintained as the result of the equilibrium between the rate at which hemoglobin is oxidized and the rate at which methemoglobin is reduced to hemoglobin. Reduction of methemoglobin is dependent upon the structural integrity of the erythrocyte, is associated with

carbohydrate metabolism and requires the regeneration of reduced pyridine nucleotides. KIESE (24) and GIBSON (12 13) have suggested that there are two pathways by which electrons can be transferred from reduced pyridine nucleotides to the ferric iron of the heme in methemoglobin. SCOTT *et al* (34) have confirmed GIBSON's postulate by demonstrating an enzyme system in normal human erythrocytes which reduced methemoglobin without requiring an artificial electron carrier Because of the preferential utilization of DPNH, this enzyme system was designated as DPNH-methemoglobin reductase or DPNH diaphorase. A severe deficiency in the activity of this system was found in the erythrocytes of many patients with hereditary methemoglobinemia (3 21 a, 29 33) Another system suggested by KIESE (24 25) and GIBSON (13) and extensively studied by HUENNEKENS *et al* (17) involves the transfer of electrons from TPNH to methylene blue or an unknown natural cofactor and then to a heme enzyme. For the same reason this system was designated as TPNH methemoglobin reductase. One patient in whom methemoglobinemia has been attributed to a hereditary deficiency in TPNH methemoglobin reductase activity has been reported (28) TOWNES *et al* (38) reported a patient with hereditary methemoglobinemia in whom methemoglobinemia has been attributed to a decreased erythrocyte GSH

The DPNH methemoglobin reductase system appears to be the major pathway by which methemoglobin is reduced in normal human erythrocytes.

It is the purpose of this study to report a Greek family with hereditary methemoglobin reductase deficiency and methemoglobinemia.

Material and Methods

Subjects. The family under study consisted of the parents and their nine children. Of these 11 individuals three sons (Nr. 3, 6 and 11 in Fig 1) were cyanotic, the cyanosis being more prominent during summer The other six children—two sons and four daughters—and the parents did not show any cyanosis. The mother's father—who died five years ago—was referred to be cyanotic when in life he was called in his village 'mchmlaris' that means in Greek 'very cyanotic

Methods. The blood samples, obtained by venipuncture, were collected in sterile tubes containing ACD. One specimen of each blood was stored 4 °C for no longer than three days prior to the performance of enzyme assays. After washing three times with 5 to 10 volumes of buffered normal saline, the red cells of another specimen were hemolyzed by the addition of 2 volumes of distilled water and 0.4 volumes of toluene. After standing at 8 °C for one hour the stroma was removed by high speed centrifugation and the resulting hemolysate was designated as 'untreated hemolysate' The oxidized

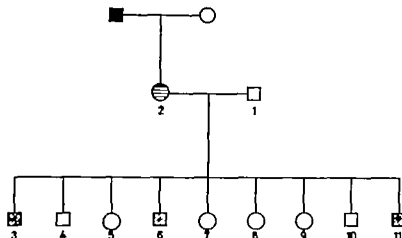


Fig. 1 Pedigree of the family reported here. The numbers 1 to 11 correspond to the numbers of Table I.

□	Male
○	Female
□ or ○	No cyanotic, no methemoglobinemic
⊠	Cyanotic and methemoglobinemic
⊖	Methemoglobinemic but not cyanotic
■	Not examined but referred to be cyanotic

hemolyzate' (presumably methgb) was obtained by incubating at room temperature (for 5 to 15 min) six volumes of the untreated hemolyzate with one or two volumes of 5% potassium ferricyanide.

Quantitative methemoglobin determination was made by using the method of EVELYN and MALLOY (6). Spectroscopic studies of the untreated and oxidized hemolyzates were carried out by using the Unicam Spectrophotometer SP 600.

Hemoglobin and methemoglobin electrophoresis was done on paper (barbital buffer pH 8.6) and in starch gel at pH 8.6 (tris-gel, tris-buffer), 7.2 (tris-gel, borate buffer) and 7.1 (tris-gel, tris buffer HCl) respectively

Glucose-6-phosphate dehydrogenase was measured by using the method of ZINKAM (40)

DPNH-methemoglobin reductase and GSSG reductase determinations were kindly performed by E. SCOTT at the Arctic Health Research Center, Anchorage, Alaska.

Results

As can be seen from Table I the father and the six children who were not cyanotic did not reveal any methemoglobinemia. Elevated levels of methemoglobin were found in the three cyanotic sons S.M. E.M. and Ch.M. In repeated measurements made within two months, the amounts of methemoglobin fluctuated between 1.32—3.8 g% 0.33—1.2 g% and 0.1—1.1 g% respectively

In the older son S M. the levels of methemoglobin from 3.8 g% lowered to 1.32 g% by the administration of two tablets of 500 mg of ascorbic acid daily for 20 consecutive days. The other two cyanotic sons E.M. and Ch.M. did not make any use of ascorbic acid or methylene blue.

Although the mother A.M. did not have detectable cyanosis she did consistently demonstrate small elevations of methemoglobin from 0 1 to 0.38 g%

Routine hematological data were within normal limits in all examined individuals except in the methemoglobinemic S.M. in whom hematocrit, hemoglobin and red cell count were found to be about 15—20% higher than normal.

Spectroscopic and electrophoretic studies of the untreated and oxidized hemolyzates by the methods described failed to demonstrate the presence of an abnormal hemoglobin.

The results of determinations of glucose-6-phosphate dehydrogenase, GSSG reductase and DPNH methemoglobin reductase activities are shown in Table I As can be seen, G-6-PD and GSSG reductase activities were within normal limits in all the examined individuals, while a deficiency of DPNH methemoglobin reductase activity could be demonstrated in the three sons S.M., E.M. and Ch.M. who had cyanosis and methemoglobinemia. In all the other individuals DPNH methemoglobin reductase activity was a little lower than normal, except the daughter B.M. in whom the enzyme activity was found to be within normal limits.

Discussion

Since 1844 more than 200 cases of probable hereditary methemoglobinemia have been recorded. Unfortunately it is not possible to determine accurately how many are the results of a deficiency in DPNH methemoglobin reductase activity and how many are due to the recently reported variants described below or to hemoglobin M. It is not unreasonable, however to assume that these patients in whom complete biochemical studies were not performed, but whose methemoglobinemia responded to the administration of methylene blue or ascorbic acid, did have an enzymatic abnormality in erythrocyte metabolism.

Although most instances of DPNH methemoglobin reductase deficiency have been described in individuals of European origin,

Table I

…oglobin concentration and G6PD DPNH-methemoglobin reductase and GSSG reductase …ctivity in lysates of erythrocytes of the family reported here.

Individual	Sex	Met-Hb gm %	G6PD activity	DNPH methemoglobin reductase activity	GSSG reductase activity	Pro… gen…
G. M. (father)	male	0	389	—	—	Mn
A. M. (mother)	female	0.1—0.38	370	0.0024	0.0100	Mn
S. M.	male	1.52—3.8	360	0.0004	0.0116	mm
H. M.	male	0	390	0.0028	0.0190	Mn
Z. M.	female	0	490	0.0023	0.0164	Mn
E. M.	male	0.33—1.2	355	0.0005	0.0124	mm
A. M.	female	0	417	0.0019	0.0148	Mn
R. M.	female	0	454	0.0042	0.0180	MN
M. M.	female	0	406	0.0023	0.0140	Mn
K. M.	male	0	359	—	—	—
Ch. M.	male	0.1—1.1	387	0.0005	0.0144	mm
…values by the methods used		0—0.3	290—600	0.0028—0.0080	0.0100—0.0240	

over 20 Alaskan Eskimos and Indians (33) two Puerto Rican (20 29) two Hindu (31) one Chinese (4) and one Cuban (7) subject with this abnormality have been reported. There are at least 37 families where more than one member was affected or where the results of biochemical studies have been compatible with a hereditary disorder

The erythrocytes of patients belonging to 35 families have been found to be deficient in DPNH methemoglobin reductase activity (3 5 21 33) Extensive investigations of erythrocytes from these patients have failed to reveal other abnormalities. The activities of various enzymes of the Embden-Meyerhof and hexose monophosphate shunt pathways and of catalase in these erythrocytes are normal (39) The findings of most family studies are compatible, with a recessive, autosomal mode of inheritance of hereditary methemoglobinemia due to DPNH methemoglobin reductase deficiency (3 21 33) Affected individuals are homozygotes and their erythrocytes contain low or absent DPNH methemoglobin reductase activity Intermediate levels of enzyme activity have been demonstrated in the erythrocytes of parents of affected children and of children of an affected parent (21 33)

Two other types of hereditary methemoglobinemia due to an enzymatic abnormality in erythrocyte metabolism are thought to exist. Müller *et al* (28) reported a patient in whom methemo-

globinemia has been attributed to a hereditary deficiency in TPNH methemoglobin reductase activity The erythrocytes of this patient contained a normal concentration of GSH. His cells were able to reduce nitrite-induced methemoglobin upon incubation with lactate, but not with glucose. Erythrocytes obtained from two brothers of this patient appeared to have the same metabolic abnormality but contained normal concentrations of methemoglobin A recessive mode of inheritance was suggested by the authors.

TOWNES and MORRISON (38) reported a case with dominant inheritance and normal TPNH and DPNH methemoglobin reductases. They demonstrated a defect in glucose utilization for methemoglobin reduction in the erythrocytes of their patient. The reduced glutathione and oxidized glutathione were also deficient. They felt that the defect with which they were dealing was one of inadequate glutathione synthesis with resultant impairment of triose phosphate dehydrogenase activity and a consequent insufficient reduction of DPN However the importance of GSH for the reduction of methemoglobin remains uncertain. Patients with hereditary absence of GSH in their erythrocytes apparently do not have methemoglobinemia.

The hereditary methemoglobinemia of the family reported in this paper is not due to the presence of any variant of hemoglobin M. The fact that electrophoretic and spectroscopic studies of all blood specimens failed to demonstrate the presence of an abnormal hemoglobin is sufficient to rule out the presence of this type of hemoglobinopathy since all the reported variants of Hb M examined in this manner have shown abnormal absorption spectra and electrophoretic pattern (1 2 8, 9 10, 11 19 22, 23 27 30, 35 37) Furthermore the presence of an abnormal hemoglobin in the family under study is also ruled out by the fact that methemoglobinemia of patient S M. responded to the administration of ascorbic acid.

From the enzymatic assays performed, it is obvious that our family has a defect in the methemoglobin reductase, which operates through the DPNH system. Actually in the three cyanotic sons in whom the levels of methemoglobin were increased, the activities of DPNH methemoglobin reductase were extremely low The daughter B.M. showed normal enzymatic activity while in all the other members of the family DPNH methemoglobin reductase activity was found to be a little lower than normal. In the mother A.M. DPNH methemoglobin reductase activity was also found to be

slightly lower than normal, although the amount of methemoglobin in her erythrocytes was found to be once above the upper normal limits. The reason for this discrepancy is not clear. It may be related to an incidentally dietary intake of methemoglobin-producing substances. A possible dietary intake of substances which act as reducing agents such as ascorbic acid might also be responsible for the transitorily found low values of methemoglobin (0.1 and 0.3 g %) while the DPNH-methemoglobin reductase activity was extremely low (0.0005) in Ch.M.'s red cells. It should be taken into consideration that the parents of the family M. are farmers cultivating mainly orange and lemon groves.

The enzymatic anomaly in our family is apparently inherited as an autosomal recessive trait. Our findings with regards to the mode of inheritance of hereditary methemoglobinemia due to DPNH methemoglobin reductase deficiency are in accordance with the data reported in the literature (3, 21, 33). Thus the three methemoglobinemic sons are homozygotes and their erythrocytes contain very low DPNH methemoglobin reductase activity. The erythrocytes of the remaining heterozygous normal children—except the daughter B.M. who is homozygous normal and has a normal enzyme activity—contain normal concentrations of methemoglobin and the DPNH methemoglobin reductase activity is nearly normal. The mother is also heterozygous and the enzyme activity of her erythrocytes is a little lower than normal. The father although not tested must also be heterozygous, otherwise the existence of homozygous affected and homozygous normal children is not compatible.

Acknowledgments. We wish to acknowledge the kind cooperation of Dr. E. Scott, Arctic Health Research Center, Anchorage, Alaska, who willingly determined the DPNH-methemoglobin reductase and GSSG reductase activities in the blood samples sent to him.

Summary

A family with hereditary methemoglobinemia due to DPNH-methemoglobin reductase deficiency is reported. Genetic studies indicate that this defect is inherited as an autosomal recessive trait. Homozygotes had cyanosis, methemoglobinemia and very low erythrocyte DPNH-methemoglobin reductase activity. Heterozygotes had no cyanosis, no methemoglobinemia (with the exception of one individual who had slight elevation of methemoglobin) and the DPNH-methemoglobin reductase activity was little lower than normal. Spectroscopic and electrophoretic studies of hemolysates demonstrated normal adult hemoglobin.

G-6-PD and GSSG reductase activities were within normal limits.

Zusammenfassung

Es wird über eine Familie mit hereditärer Methämoglobinämie infolge eines Mangels an DPNH-Methämoglobinreduktase berichtet. Genetische Untersuchungen ergaben, daß der Defekt autosomal rezessiv vererbt wird. Homozygote Individuen hatten eine Zyanose, eine Methämoglobinämie und eine sehr niedrige Aktivität der DPNH-Methämoglobinreduktase. Heterozygote Individuen wiesen keine Zyanose und keine Methämoglobinämie auf (mit Ausnahme eines Probanden mit einer geringen Vermehrung von Methämoglobin) und die Aktivität der DPNH Methämoglobinreduktase war etwas niedriger als normal. Spektroskopische und elektrophoretische Untersuchungen von Hämolysaten ergaben normales Hämoglobin A. Die Aktivitäten der Glukose-6-phosphatdehydrogenase und der Glutathionreduktase waren im Bereich der Norm.

Résumé

Un rapport sur une famille atteinte de methémoglobinémie héréditaire due à une déficience en DPNH-methémoglobine-réductase est présenté. Les études génétiques réfèrent que cette déficience est héritée selon un mode autosomal récessif. Les personnes homozygotes avaient une cyanose, une methémoglobinémie et une activité très basse de la DPNH-methémoglobine-réductase. Les personnes hétérozygotes n'avaient pas de cyanose ni de methémoglobinémie (à l'exception d'une personne ayant une très faible augmentation de méthémoglobine) et l'activité de la DPNH-methémoglobine-réductase était un peu plus faible que normalement. Les examens spectroscopiques et électrophorétiques d'hémolysats mirent en évidence de l'hémoglobine A normale. L'activité de la G-6-PD et de la glutathion-réductase était dans les limites de la normale.

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Metabolism of Leukocytes in a Case of Acute Leukemia*

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The concept of rapid proliferation of primitive cells in acute leukemia has come under increasing criticism. Despite the finding of increased nucleic acid metabolism in leukemia by chemical methods when compared to normal marrow (1–3) the actual generation time of the leukemic blast has been found to be as long or longer than neutrophilic precursors (4). GAVOSTO *et al.* (5) have shown by means of high resolution autoradiography that leukemic blast cells have a decreased proliferative capacity for deoxyribonucleic acid synthesis when compared to normal blast cells. Furthermore, LAJTHA (6) has demonstrated a decreased rate of DNA synthesis between leukemic blasts and normal myelocytes, but made no such comparison with normal blasts because of statistical pitfalls. CRADDOCK and NAKAI (7) have referred to a decreased rate of DNA synthesis in leukemic blasts, but presented no data on normal cells. Recently SCHUMACHER and SALEN (8) utilizing autoradiography and liquid scintillation studies have observed that most patients in the terminal phase of acute leukemia manifested peripheral blast cell populations with decreased tritiated thymidine incorporation.

Since it is well established that cells entering mitosis are more susceptible to destruction (9) the decreased proliferative capacity for deoxyribonucleic acid synthesis could explain drug resistance and failure of therapy at this stage of leukemia. This report deals with an attempt to stimulate DNA synthesis in an effort to produce a more vulnerable cell population.

This investigation was supported in part by Public Health Research Grant CA 08245–01 and application number 3576 from the American Cancer Society

Methods and Materials

The techniques for liquid scintillation counting and autoradiography have appeared in detail in a previous publication (6). The tritiated thymidine and uridine used in liquid scintillation studies had specific activities of 3.0 C/mM and 1.15 C/mM respectively. The tritiated thymidine used for autoradiography had a specific activity of 3.0 C/mM.

The vitamin B_{12} and folic acid were administered by intramuscular injection. Vincristine was given by the intravenous route (Eli Lilly and Company, Indianapolis, Ind.). Prednisone and 6-mercaptopurine were given orally. The methotrexate was administered by oral and intravenous route.

Case Report

The diagnosis acute lymphatic leukemia in a 12 year old white girl, was established by peripheral blood studies and bone marrow examination in November 1962. The bone marrow was completely replaced by lymphoblasts. At that time, a partial remission was induced after oral therapy with 6-mercaptopurine, methotrexate, and prednisone. Over the next 14 months, her leukemic symptoms were controlled with repeated courses of 6-mercaptopurine, methotrexate and symptomatic care. When symptoms and signs of relapse were apparent, she was admitted to Jefferson Medical College Hospital in February 1964.

At the time of admission the patient was acutely ill with an oral temperature of 104°F. There was marked pallor of the mucous membranes, ulceration of the buccal membrane, but no significant lymphadenopathy or hepatosplenomegaly. Leukocytes 17000/mm³ with 50% primitive cells and 40% mature lymphocytes. The marrow was entirely replaced by lymphoblasts. Treatment was initiated with intravenous vincristine (0.75 mg, 2 mg/M²). The *in vitro* autoradiography and liquid scintillation studies utilizing tritiated thymidine and uridine were started at this time.

Results

The incorporation of tritiated thymidine and uridine into the blast cell population showed marked variability (Fig 1). The number of blasts present show definite inverse correlation with the incorporation of tritiated thymidine ($r = -0.84$ significant at $<1\%$ level) and tritiated uridine ($r = -0.97$ significant at $<1\%$ level). As the population of blasts diminished, the ability of those remaining to incorporate thymidine greatly increased. Simultaneous autoradiography (Table I) performed at times of high and low thymidine and uridine incorporation was in agreement with the above findings. Labelling in 4 normal controls by our techniques demonstrated a mean level of 0.01%. The cells demonstrating labelling were usually monocytes and lymphocytes.

The patient was discharged from the hospital on March 3, and observed at weekly intervals. At the end of 4 weeks, vincristine therapy was discontinued because no further clinical improvement was seen. Her leukocyte count was 15000/mm³ with 70% primitive

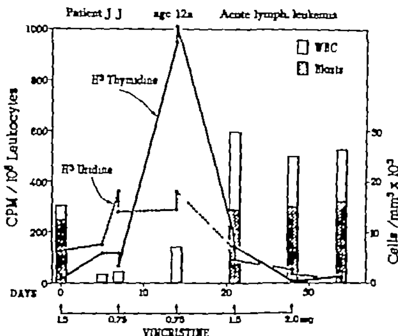


Fig 1 Liquid scintillation studies in relation to the blast cell count and treatment. Specific activity — tritiated thymidine 3.0 C/mM tritiated uridine 1 15 C/m M. (White blood cell counts and percentage of blast cells were determined from the peripheral blood smears and not the autoradiographic slides.)

Table I

Comparison of autoradiography and liquid scintillation data performed at highest and one of lowest points of tritiated thymidine incorporation.

Date	Percent of total cells, blasts unlabeled	Percent of total cells, blasts labeled	Average grains per labeled cells	Counts per min/10^8 Leukocytes (Liquid Scintillation counting)
*3/6/64	17 1	5.1	15.1	995
3/20/64	59.0	0.00	0.00	2.8

Percentage of blasts much higher here than observed in usual peripheral blood smears. Due to technique in preparation.

cells and 20 % lymphocytes. From March 15, 1964, to May 3, 1964, she received oral 6-mercaptopurine and intravenous methotrexate. Because of the progression of the leukemic state, studies were again begun on May 3, 1964 Her physical findings included an oral temperature of 102 °F numerous petechiae over the dependent portions of the body and marked pallor of the mucous membrane. Leukocytes 30,000/mm³ with 90% primitive cells and 10 lymphocytes. Repeat bone marrow examination revealed that the marrow was entirely replaced by lymphoblasts. Beginning May 4, 1964, (Fig. 2)) folic acid and vitamin B_{12} were administered in an attempt to stimulate nucleic acid synthesis and vincristine was utilized to destroy the deoxyribonucleic acid synthesizing

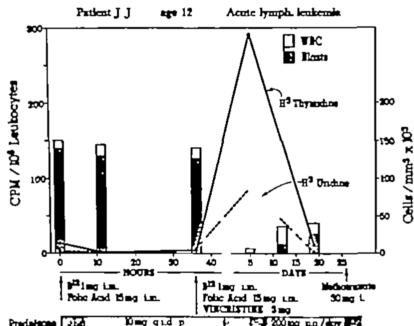


Fig 2. Liquid scintillation studies in relation to the blast cell count and attempted stimulation therapy Specific activity — tritiated thymidine 3.0 C/mM tritiated uridine 1 15 C/mM. (White blood cell counts and percentage of blast cells were determined from the peripheral blood smears and not the autoradiographic slides.)

vulnerable cells. Twelve and 36 hours after the administration of these agents no increase in the incorporation of tritiated thymidine or uridine was found. However following the administration of vincristine and increased prednisone, the total leukocyte and blast count decreased markedly The remaining cells showed increased ability to incorporate tritiated thymidine and uridine. After short clinical and hematological improvement for few days her clinical and hematological condition gradually deteriorated and she expired on June 18, 1964

Discussion

By serial studies, it was evident that there was great variability in the incorporation of nucleosides into peripheral leukemic cells at various times in the patient's illness. RUBINI *et al.* (10) in an *in vitro* autoradiography study on peripheral leukocytes of patients with non-hematologic disorders, viral disease, and blood dyscrasias, which included leukemia, showed such a variability within these groups. Our patient showed decreased incorporation of the nucleotides during the terminal phase in the peripheral leukocytes. The attempt to stimulate the leukemic cells into a vulnerable period of

DNA synthesis with folic acid and vitamin B_{12} appeared to be ineffective by our measurements. Yet, following administration of vincristine and steroids in large doses, there was a precipitous decrease in the number of cells. The mechanism of this effect was not clear since large doses of steroids alone have been reported on occasion to cause this effect (11). It should be emphasized that the mechanism was probably not related to a stathmokinetic effect of vincristine (12) since the cells were not synthesizing DNA and were not in mitosis. It would also appear that treatment of these metabolically inactive cells with other antimetabolites would fail in this stage since the action of 6-mercaptopurine and methotrexate depends on nucleotide synthesis (13–14). BOND *et al.* (15) utilizing *in vitro* autoradiographic studies were unable to depress tritiated thymidine incorporation of acute leukemic leukocytes in short term incubation by antimetabolites. However they suggested that short term culture techniques with antimetabolites might demonstrate some effect on tritiated thymidine utilization by leukemic cells.

The decreased incorporation of tritiated thymidine and uridine in this patient's leukocytes was only observed in the periphery and does not account for the bone marrow activity which would be extremely important in regard to stimulation therapy. MAUER and FISHER (16) have shown by *in vivo* labelling with tritiated thymidine and subsequent autoradiographic studies that peripheral blood and bone marrow vary in labelling. In general, their patient showed a lower percentage of blasts labelled in the peripheral blood than in the bone marrow. KILLMAN *et al.* (4) reported one patient with acute myeloblastic leukemia who had a small number of highly labelled cells in the bone marrow but no labelled cells in the peripheral blood. We have observed an adult patient with terminal acute lymphoblastic leukemia with similar labelling in blood and bone marrow and four of five patients with acute leukemia had decreased incorporation of tritiated thymidine by peripheral blasts in the terminal stage (8). From this sparse data no conclusion concerning the relationship between blood and bone marrow nucleoside incorporation can be drawn. Unfortunately in our case tritiated thymidine and uridine were not incubated with the bone marrow cells.

Acknowledgement. The authors are indebted to Miss BEVERLY KIRCHNER of York Hospital for her aid in the preparation of the manuscript.

Summary

Incorporation of tritiated thymidine and uridine by peripheral acute lymphocytic leukocytes were studied by means of autoradiography and liquid scintillation counting. The blast cells in the terminal stage showed marked decrease incorporation of both nucleosides. An attempt to stimulate nucleic acid synthesis with folic acid and vitamin B_{12} and then block by means of vincristine and steroids was unsuccessful. Nevertheless, the procedure is presented as a possible trial in the terminal phase of acute leukemia.

Zusammenfassung

Der Einbau von Tritium-markiertem Thymidin und Uridin in die peripheren Leukozyten einer akuten Lymphadenose wurde untersucht mit Hilfe der Autoradiographie und der Flüssigkeits-Szintillationszählung. Im terminalen Stadium zeigten die Blasten eine deutliche Verminderung der Inkorporation beider Nukleoside. Der Versuch, die Nukleinsäuresynthese mit Folsäure und Vitamin B_{12} zu stimulieren und dann mit Vincristin und Steroiden zu blockieren, misslang. Trotzdem wird dieses Vorgehen als möglicher therapeutischer Versuch in der terminalen Phase einer akuten Leukämie dargestellt.

Résumé

L'incorporation de thymidine et d'uridine tritiée par les leucocytes d'une leucémie lymphatique aiguë a été étudiée par autoradiographie et à l'aide d'un compteur à scintillateur liquide. Au stade terminal, les blastes présentèrent une incorporation nettement diminuée des deux nucléosides. Un essai de stimuler la synthèse des acides nucléiques à l'aide d'acide folique et de vitamine B_{12} et de la bloquer en suite par de la vincristine et des stéroïdes échoua. Ce procédé n'en est pas moins présenté en tant qu'essai thérapeutique possible dans la phase terminale d'une leucémie aiguë.

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Early Appearance of Blast Like Cells in the Thoracic Duct Lymph of Rats Given Bacterial Endotoxins

Gisela Hammerli and M. Landy

It is generally accepted that the cellular composition of the thoracic duct lymph in rodents consists of at least 95–97% small lymphocytes and that the remainder is made up of larger mononuclear elements (1, 2). During the course of studies on the appearance of tumor cells in the thoracic duct lymph of rats bearing Walker ascites tumor, it was found that the proportion of these larger mononuclear cells was higher than that in normal animals (3). This finding was interpreted as probably representing a nonspecific response. Accordingly, tests were made with India Ink as an inorganic, colloidal, purely physical agent. Unexpectedly, the product used (Günther Wagner, Hannover) evoked a similar effect. Eventually it was ascertained that this effect of India Ink was due to contamination with Gram negative bacteria. Thereafter experiments were conducted with purified endotoxins, derived from various Gram-negative species; these bacterial components were found to be by far the most effective agents in evoking the appearance of blast like cells in thoracic duct lymph. This communication reports experiments on factors such as individual endotoxin preparations, dosage, timing, etc. which determine the magnitude of this distinctive cellular response in rats; some cytological characteristics of the blast-like cells are also described.

Material and Methods

Cannulation of the thoracic duct was performed on 160 random-bred, conventionally raised CFN rats by means of a modification of Reinhardt's method (3, 4). The general design of the experiments, including the number of animals, the time of cannulation and the dosage of endotoxin (dissolved in Ringer) is given in Table I. The Gram-

Table II

Induction by endotoxin of enlarged thoracic duct cells in rats effect of dose.

S. Enteritidis endotoxin (μg)	Medium-sized cells 7.2 10.2 μm (in %) 1 h	3 h	24 h	Large-sized cells more than 10.3 μm (in %) 1 h	3 h	24 h
0.0001	5.6±1.3	4.7±0.9	5.0±0.7	0.1	0.1	—
0.001	6.4±1 4	6.1±1.3	5.1±1.2	—	—	—
0.01	9 1±1.2	10.1±2.4	9.7±2.5	0.1	0.2	0.2
0.1	10.1±1.8	11.0±1 7	9.8±2.9	0.1	0.4	0.4
1 0	10.6±1 1	8.9±2.2	9.8±2.2	0.1	—	0.1
10.0	10.5±1.8	9.7±1.6	8.6±0.4	0.5	0.3	0.3
100.0	17 4±2.3	14.8±1.8	12.3±1.8	1.3	0.4	0.2
Ringer solution	5.3±1 4	5.9±0.7	5.3±2.2	—	—	—
Controls	—	4 6±0.8	—	—	—	—

Table III

Induction by endotoxin of enlarged thoracic duct cells in rats kinetics of the effect.

Stimulation	Cannulation (hours after injection)	Medium-sized cells 7.2 10.3 μm (%)	Large-sized cells more than 10.3 μm (%)
S. Enteritidis endotoxin 100 μg	1	17 4±2.3	1.3±0.3
	3	14.8±1.8	0.4
	24	12.3±1.8	0.2
	72	7.0±1.9	0.1
	96	5.8±1 1	—
	120	5.4±1 1	—

was performed 72, 96 and 120 h after endotoxin had been given. The results of the experiments, given in Table III show that most of the medium and large-sized cells appeared within one hour after the injection. As the interval between injection and sampling increased, their number gradually diminished and by 96 h, the lymph cell composition was once again similar to that of non-stimulated animals.

To ascertain whether the effect obtained was typical of bacterial endotoxins generally somatic lipopolysaccharides derived from *Shigella flexneri Escherichia coli* and *Serratia marcescens* were also examined. The results of experiments in which rats were given 100 μg of endotoxin from these other Gram negative genera and cannulated one hour later are summarized in Table IV Values for the medium and larger-sized cells were within the same range for the various compounds employed.

Table IV

Induction by endotoxin of enlarged thoracic duct cells in rats comparison of products from different genera.

Endotoxin (100 µg) derived from	Medium-sized cells 7.2 10.3 µm (in %)	Large-sized cells more than 10.3 µm (in %)
Salmonella enteritidis	17 4 ± 2.3	1.3 ± 0.5
Escherichia coli	16.7 ± 3.0	0.7 ± 0.7
Serratia marcescens	16.9 ± 2.9	1.2 ± 1.0
Shigella Flexneri	16.3 ± 2.0	0 8 ± 0.4

Staining characteristics and DNA content. Fig 1 and 2 show blast like cells in the supravital state under phase contrast. In smears, most of these cells had a large delicately structured nucleus and a small rim of cytoplasm which stained deep-blue with Giemsa (Fig 3) bright red with methylgreen-pyronin and which exhibited a brilliant red fluorescence after treatment with acridine orange. Nucleoli were inconspicuous however staining with methylen blue evealed several spot like nucleoli Acid and alkaline phosphatase and non-specific esterase could not be demonstrated in these ele ments. Apart from the blast-like cells, a few with one or two large nucleoli and a more abundant pale-staining cytoplasm were obser ved, also various numbers of typical monocytes.

The relative DNA content in Feulgen-stained nuclei of normal rat sperm cells and of small lymphocytes, presented in Fig 4 was found to be around c and 2c respectively and thus corresponds to the known haploid and diploid chromosome number of these cells Medium-sized and large lymphoid cells showed DNA values which accumulated in a peak around 2c and then stayed at a certain level up to the 4c amount. It can be assumed that the cells aggregated around 2c are in the Gl-phase, and that those with higher amounts of DNA are in the S-phase. A second peak, characteristic for a di- iding population, was not striking in the frequency distribution of the larger lymphoid elements.

Discussion

In rats given a single intraperitoneal injection of bacterial endotoxin, the thoracic duct lymph contains within one hour besides a higher amo nt of small lymphocytes a very substantial number of medium- and large-sized mononuclear cells. As regards size mor phological features and staining reactions, these elements can be divided into 3 morphological classes. The majority consist of

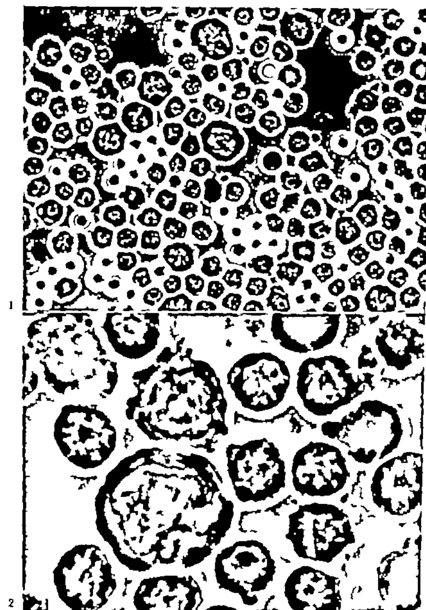


Fig. 1. Blast-like cells in the thoracic duct-lymph of rats given 100 μg *S. enteritidis* endotoxin i.p. for one hour. Phase contrast 480:1.

Fig. 2. Blast-like cells in the thoracic duct-lymph of rats given 100 μg *S. enteritidis* endotoxin i.p. for one hour. Phase contrast 1250:1.

blast like basophilic cells. In addition, there are discerned a few cells with a paler staining cytoplasm, presumably precursors of medium lymphocytes, and monocytes as well.

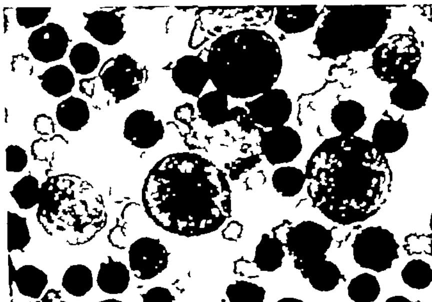


Fig. 3. Blast-like cells in the thoracic duct-lymph of rats given 100 μg *S. enteritidis* endotoxin i.p. for one hour. Giemsa 1250:1

The DNA values of the large and medium-sized cells range from 2c to 4c indicating that these cells are synthesizing DNA. As no metaphases could be found, it cannot be ascertained whether the cells in the 4c range are in the G2 phase after having completed their synthesis, or whether they have entered the mitotic cycle. Correlative studies of DNA measurements and autoradiography with H³ thymidine on human thoracic duct lymphocytes by Cooper *et al.* (10) also demonstrated a DNA-synthesis of the 0.2–0.5 % medium- and large lymph cells present. Cultivation in the presence of phytohemagglutinin caused a considerable increase in the percentage of these cells.

Two features characterize the blast like cells which appear in the thoracic duct lymph after stimulation with endotoxin. In contrast to the large cells which develop from small lymphocytes in tissue culture in the presence of various stimuli and in opposition to the large pyroninophilic cells observed in the lymphatic tissues of animals after antigenic stimulation, the large basophilic cells in the thoracic duct lymph do not have conspicuous nucleoli, and

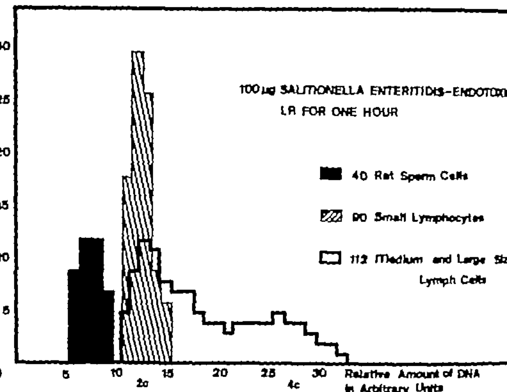


Fig 4 Relative amount of DNA in Feulgen-stained nuclei of thoracic duct cells in rats given bacterial endotoxin.

moreover their emergence occurs with great rapidity Under tissue culture conditions employed by most workers, cellular transformation generally is seen only after 24 h or more. The earliest changes of human peripheral leukocytes in PHA cultures were observed by COOPER *et al* (10) who reported that large mononuclear cells increased in size after having been in culture for 5 h. RIEKE *et al* (11) who appear to be the only authors studying the stimulation of rat thoracic duct cells, described an increased amount of large lymphocytes 1–14 days after stimulation with pertussis vaccine and irradiation.

The early appearance of the large, blast like cells raises the compelling issues of their origin and their functional significance As to the former every organ tributary to the thoracic duct and containing lymphatic tissue, can be considered as a possible source of these elements Assuming that intraperitoneally injected endotoxin rapidly accumulates in these sites, two possible explanations

come to mind to account for the release of the blast like cells into the thoracic duct (1) an emergence of pre-existing cells, (2) formation by division of stem-cells. In view of the marked rapidity of the effect, the second possibility seems rather unlikely. It is therefore more reasonable to assume that these elements represent pre-existing cells, emerging from lymphatic tissue under the influence of the agents administered. However we were unable to find appropriate numbers of corresponding cells with regularity in sections of mesenteric, cisternal and mediastinal lymph nodes which are located in the area draining the thoracic duct. Work is now in progress to determine the origin of these cells by means of H³ autoradiography.

In seeking to establish the basis for this phenomenon it will be important to determine whether (a) these cellular events are peculiar to bacterial endotoxins or whether they can also be evoked by other high molecular weight polysaccharides, and (b) in the case of bacterial endotoxins, whether the changes are attributable to their pharmacologic or to their immunologic properties. Although the possibility of immunologic factors cannot at present be entirely excluded, there are reasons for believing that a pharmacological mechanism is the more likely. Studies are in progress in which experiments have been designed to resolve these issues.

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Summary

A substantially heightened amount of large mononuclear cells appeared within one hour in the thoracic duct lymph of rats after the intraperitoneal injection of purified endotoxin from Salmonella enteritidis. DNA measurements showed them to synthesize DNA. Experiments are under way to determine origin and function of the blast-like elements.

Zusammenfassung

Eine beträchtlich erhöhte Zahl großer mononukleärer Zellen fand sich innerhalb einer Stunde in der Lymphe des Ductus thoracicus von Ratten, die intraperitoneal mit gereinigtem Endotoxin von Salmonella enteritidis injiziert waren. Diese Blasten-ähnlichen Elemente synthetisieren DNS. Untersuchungen über ihre Herkunft und Funktion sind im Gange.

Table I

Serum from hen A which was repeatedly immunized with washed red cells from the hens B C and D Tube technique, equal volumes of serum and 2 per cent suspension of red cells in saline. Incubation time 1½ hour The sera were either active or inactivated t 56 C for 30 min to destroy complement.

Serum	Tested against erythrocytes from hen A			B		
	37	20	4	37	20	4
A serum	—	—	—	++++	+++	+++
A serum inact.	—	—	—	++++	++++	+++
D serum	—	n. t.	—	—	n. t.	—
D serum inact	—	n. t	—	—	n. t.	—

t. not tested

cluded chicks inoculated with virus at the age of 21 days which usually results in a high incidence of subleukemic anemia. Finally one group was virus-inoculated at the age of 70 days. At that age the chicks are resistant to virus infection and do not develop either leukemia or anemia

Material and Methods

Fowl strain. A strain of White Leghorn (Edo) was used. The characteristics and maintenance of this strain have been reported earlier (4)

Virus strain. The strain of myeloblastosis virus (BAI-strain) and the preparation of virus suspensions have been described elsewhere (4). Each chick received intravenously 0 5 ml heparinized plasma from young leukemic chicks. This plasma had been lyophilized and stored frozen for 1 2 years. According to ATP-ase determinations, the selected plasma would produce leukemia in 70–80% of newly hatched chicks (4).

Hematocrit determinations were performed with the micromethod, using heparinized capillary tubes and reading after centrifuging for 5 min in hematocrit centrifuge (International eq comp.)

The occurrence of leukemia was determined in tw way The peripheral blood picture was checked in smears stained by the MA -G UNWALD-GIEMSA method and microscopical studies were made on tissues from birds dying or being killed during the course of the investigations. Pieces of bone marrow liver spleen and *bursa Fabricii* were prepared for microscopy and the sections were stained with haematoxylin and eosin or by V AN GIESON technique. Sections from the spleen were also stained by H method for iron pigment.

Fowl anti-erythrocyte serum of blocking type Saline glutinins were produced in hen by immunization with pooled red cells from three hens (Table I) The saline agglutinins of this serum were inactivated by treating 1 5 dilution of the serum for 15 min at temperatur of 69 °C (Cf) After this treatment the serum did not agglutinate red cells from different hens using the same technique as that given in *Table I* These cells, however in 2 suspension after being washed carefully three times reacted with the RAFA serum (see below Controls w th washed red cells and RAFA were negative.

Table I (continued)

Serum	Tested against erythrocytes from hen C 37	C 20	C 4	D 37	D 20	D 4
A serum	++	++	++	+	++	++
A serum inact.	+++	+++	+++	+	++	+
D serum	—	n. t.	—	—	n. t.	—
D serum inact.	—	n. t.	—	—	n. t.	—

The titre of the blocking antibody using the RAFA technique was about 1 100 compared with 1 32 for the agglutinating antibody

Rabbit anti-fowl serum was prepared by repeated immunization of rabbit with pooled hen serum. After absorption with carefully washed red cells this serum was used throughout the investigation as absorbed serum, RAFA. The properties of the sera are shown in Table II.

Direct Coombs' test. A 2% suspension of red cells washed at least three times was incubated with equal volume of RAFA serum diluted 1 40 with saline for 45 min t room temperature. Readings were made after short centrifugation at low speed. The supernatant was always checked for RAFA activity with sensitized red cells (i. e. red cells washed three times and coated with the blocking antibody) Such activity could always be demonstrated.

Control of non-specific positive tests. The influence of protein concentration and changes of activity of RAFA has continuously been controlled. Experiments were performed which showed that weak positive reactions could appear with direct Coombs' test in blood stored cold. T avoid such technical errors, the tests in the main experiments were always performed on fresh blood which had not been stored cold.

Experimental groups. Sixteen chicks were inoculated with virus when 9 days old. Twenty chicks were inoculated at the age of 21 days and 6 chicks at the age of 70 days. Nine surviving chicks in the 21-day-old group received new dose of virus after 27 days. Every second day all chicks were checked hematologically and serologically according to the methods described above.

The chicks inoculated when 21 or 70 days old were followed for 60 days after virus inoculation.

Four chicks not virus inoculated served as controls in the 21-day-old group. The serological tests were always done as blind tests, and anonymous samples from the four controls were always among the samples from the test-chicks. I addition, the direct Coombs' test has been performed on the blood from several uninoculated chicks of different ages. All these control tests were negative.

Results

Hematological Findings

The results of these investigations are presented in Fig 1 2 and 3

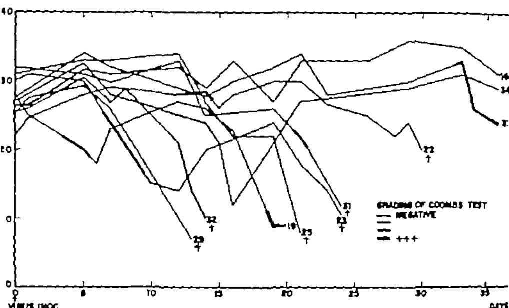


Fig 1 Serological ('direct Coombs test') and hematological behaviour of chicks after virus inoculation at 9 days' age. The direct Coombs test was graded as — to +++ as indicated in the figure. The crosses indicate that the animal died or was killed when moribund.

Chicks inoculated when 9 days old. Only one of these chicks (No 19) developed the classical form of the leukemia (Fig 1) Six out of the 16 inoculated chicks (No 22, 23 25 29 31 and 32) became anemic. Microscopical examination of the anemic chicks revealed findings typical for subleukemic anemia, the most prominent features of which are lymphoid hyperplasia and small necrotic foci in a subleukemic marrow together with splenomegaly and splenic siderosis (4)

Chicks inoculated when 21 days old The incidence of classical leukemia was 10% in this group (Table III) Two chicks (No 64 and 73) had the disease course characteristic of subleukemic anemia and died with severe anemia 16 and 23 days after virus inoculation (Fig 2) These were the only chicks in this group with definite subleukemic anemia although some of the other chicks had slightly decreased hematocrit values.

Chicks inoculated when 70 days old. None of the chicks inoculated at 10 weeks of age developed leukemia. One chick (No 6) had a short episode of anemia around the 10th day after virus inoculation

Table II
Preparation of rabbit anti-fowl serum and absorbed anti-fowl serum (RAFA)

Rabbit anti-fowl serum	Tested against erythrocytes from hen B	RAFA	Tested against sensitized erythrocytes from hen B
Undil.	+++	Undil.	—
1 2	+++	1:2	+
1 4	+	1 4	+
1:8	—	1 8	++
		1:16	+++
RAFA		1:32	+++
undil.	—	1 64	+++
		1:128	+
		1 256	—

A rabbit was repeatedly injected with pooled serum from ten hens. The serum from the rabbit was then inactivated t 56°C for 30 min. This inactivated serum was bsorbed with carefully washed red cells from the hen B. The ratio of 4 volumes of the serum and 1 volume of red cells from hen B were used for bsorption for one hour t room temperature. This absorbed serum is called RAFA. Tube technique, equal volumes of 2% cell suspension and serum were used. Room temperature, 45 min, reading after slight centrifugation.

Red cells from hen B were incubated with blocking antibodies from hen A serum for 1 hour and then washed three times.

Table III
Summary of the hematological and serological findings after inoculation of chicks with myeloblastosis virus.

Age at inoc.	No. classical leukemia	No. subleukemic anemia	No. positive direct Coombs' reactions	No. of all inoc. showing at least 10 units decrease in hematocrit	No. of Coombs' positive chicks showing t least 10 units decrease in hematocrit
9 days	1/16	6/16	8/16	10/16	8/8
21 days	2/20	2/20	7/20	6/20	2/7
70 days	0/6	0/6	0/6	1/6	0/0

(Fig. 3) The anemia regressed, however and at the end of the observation period i. e. after 29 days, all animals were hematologically normal.

Serological Findings

Chicks inoculated when 9 days old. Eight of the 16 chicks inoculated at one week of age had positive direct Coombs' reactions. The first positive reaction was observed as early as one day after the virus inoculation (Fig 1). Most of the positive reactions occurred very irregularly and there was a different grading of the reactions on

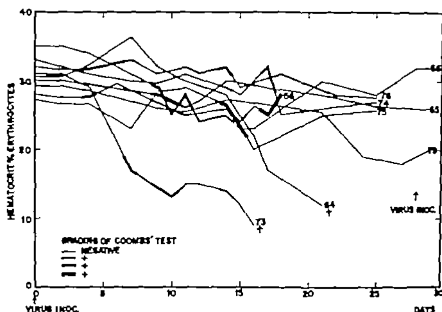


Fig. 2. Serological ('direct Coombs' test') and hematological behaviour of chicks after virus inoculation at 21 days' age. The direct Coombs' test was graded as — to +++ as indicated in the figure. The crosses indicate that the animal died or was killed when moribund.

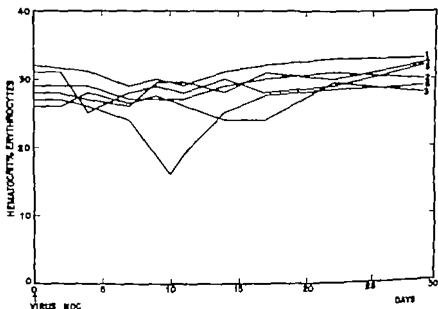


Fig. 3. Hematological behaviour of chicks after virus inoculation at 70 days' age. No direct Coombs' test was positive in these chicks.

successive days in some of the chicks. All chicks having positive Coombs' tests also had a decreased hematocrit, and in most of the chicks there was a direct association between the positive Coombs' reactions and the decrease of the hematocrit values.

Two of the chicks differed from the others by becoming serologically positive during a later time period. Chick No 33 became positive 33 days after the inoculation, and was killed on day 36 when the erythrocytes gave a strongly positive direct Coombs' reaction (+++) The indirect Coombs' test on the serum of this chick tested against erythrocytes from another hen was negative.

The erythrocytes of another chick (No 34) gave a slightly positive Coombs' reaction (+) several times during the periods 12–14 and 41–57 days after the virus inoculation. The last period is not included in Fig 1 There was no actual anemia. The chick was killed on day 66 and there was no microscopical evidence of leukemia.

One chick (No 29) developed a rapidly progressive subleukemic anemia and died 13 days after virus inoculation without being serologically positive during any period of the disease.

Chicks inoculated when 21 days old. As can be seen in Fig 2 and Table III seven of the chicks inoculated with virus at 21 days of age had positive direct Coombs' reactions for periods of different durations. These occurred during a limited period, ranging from day 4 to day 22 after the virus inoculation.

As can be seen in Fig 2 there was quite a different grading of the tests on successive days in many of the chicks. Unlike the results for the foregoing group there was no obvious association between positive Coombs' reactions and decrease of the hematocrit value, and one chick (No. 64) died in a profound anemia 22 days after virus inoculation without being serologically positive during any period of the disease. As is seen in Table III only two of the seven chicks with positive Coombs' reactions had more than a 10-unit decrease in their hematocrit values.

In order to check viral influence on the serological reaction, 9 surviving animals were inoculated with more virus 27 days following the start of the experiments. The direct Coombs' tests during the following two days were all negative.

Chick No. 56 was killed on the 18th day after the first virus inoculation when the direct Coombs' test was strongly positive (+++) The chick was killed by exsanguination and the serum

was tested for the presence of erythrocyte antibodies with the indirect Coombs test method. This test was negative.

Chicks inoculated when 70 days old. None of the chicks which were inoculated with virus at 70 days of age had positive serological tests.

Controls All the direct Coombs' tests on fresh blood from chicks which had not been virus inoculated were negative.

Discussion

The investigations have demonstrated that many of the chicks inoculated with the myeloblastosis virus when 9 and 21 days old became serologically positive to direct Coombs' tests on the erythrocytes. The serological behaviour in individual chicks after virus inoculation underwent great variation. Strongly positive reactions could change to negative reactions the following day (No. 75) and the reactions could alternate between positive and negative as in chicks No 34 56 73 74 and 76 If the positive direct Coombs reactions were the result of an (auto)immunization process, they would be more stable. One would also presume that alternation from strongly positive to negative reactions would be associated with hemolytic crises. In some of the chicks the positive Coombs' test was actually associated with a decrease in the hematocrit value but in many of the chicks this was not the case. The appearance of a positive direct Coombs reaction in one of the chicks in the 9-day-old group one day after virus inoculation also indicates that the reaction was not caused by antibodies.

From day 4 on, there were several positive reactions among the chicks inoculated at the age of 9 and 21 days. The period of 4 days from virus inoculation until the first positive reaction seems to be remarkably short for developing antibodies. Furthermore the strongly positive reactions seen in these experiments would persist for a longer period if antibody dependent.

The above mentioned phenomena indicate that the positive direct Coombs' reactions were not caused by (auto)antibodies. The negative indirect Coombs reactions also exclude free antibodies. It seems therefore, reasonable to suggest an association with the leukemia or the leukemia virus. An association with the leukemia is indicated by the fact that the positive reactions appeared only in the group of 9 and 21-day-old chicks, but not in the 70-day-old chicks

which are resistant to virus infection at that age. On the other hand the negative reactions in the oldest group might depend on the chicks being more mature immunologically and therefore able to react to the virus infection with production of viral antibodies to prevent viremia. The fact that most of the positive reactions in the younger groups occurred during the period from 4 days after the virus inoculation is consistent with the time of virus production in the chick.

However some of the positive direct Coombs' reactions occurred in chicks not developing leukemia or subleukemic anemia. If the reactions depended upon the presence of virus, it would indicate virus production without a tumour producing effect of the virus.

Experiments were also performed to determine whether incubating virus-containing serum and fowl erythrocytes *in vitro* for two hours at 37 C could result in a change in the serological reactivity of the cells detectable by the direct Coombs test. All these tests were negative. This indicates that plasma proteins were not attached to the red cells by virus influence.

In an additional experiment, a virus suspension was washed free from surrounding plasma by 4 washings in a Spinco ultracentrifuge for 30 min at 30 000 rpm. The washed virus pellet did not absorb antibodies from RAFA serum. This showed that the virus itself does not combine with plasma proteins.

The early and irregular appearance of positive direct Coombs reactions and the absence of a definite association with the anemia thus indicate that the positive serological reaction and the subleukemic anemia are not caused by an immunization process. The *in vitro* experiments have shown that virus particles do not become attached to the red cell surface and cause positive serological reactions under the prevailing conditions. However the fact that the positive serological reactions occurred only in virus-inoculated animals seems nevertheless to suggest an association with the occurrence of virus.

The negative serological results for our control chicks do not fit in with the results obtained by WARNER who could demonstrate pseudo-Coombs positive reactions in normal chicks (6) The discrepancy is probably due to differences in the rabbit anti fowl serum employed by the respective investigators, although differences in other methodological procedures might also be responsible.

Summary

In order to evaluate whether the atypical course of myeloid leukemia, the subleukemic anemia, has an immunological etiology chicks were inoculated with myeloblastosis virus and investigated hematologically and serologically including reaction with a rabbit anti-fowl serum Coombs' test. Three age groups were studied, chicks 9, 21 and 70 days old at the time of virus inoculation. Fifty and 35% of the chicks inoculated 9 and 21 days old, respectively developed positive direct Coombs' reactions. No positive reactions were observed in the 70-day-old group. The serological reactions occurred very irregularly and there was no definite association between the direct Coombs' reaction and the anemia. The findings indicate that the subleukemic anemia and the positive serological reactions were not dependent on auto-antibodies. They suggest that the anemia *is instead due to an association of virus particles with the erythrocytes.*

Zusammenfassung

Um nachzuweisen, ob der atypische Verlauf der myeloischen Leukämie, die subleukämische Anämie immunologisch bedingt ist, wurden Küken mit dem Virus der Myeloblastose inokuliert und hämatologisch und serologisch untersucht einschließlich des Coombs-Testes mit Anti-Huhn-Serum des Kaninchens. Es wurden drei Altersgruppen untersucht: Küken, die zur Zeit der Inokulation 9 21 und 70 Tage alt waren. Von den im Alter von 9 und 21 Tagen inokulierten Küken zeigten 50 bzw 35% einen positiven Coombs Test. In der 70 Tage alten Gruppe fanden sich keine positiven Reaktionen. Die serologischen Reaktionen traten sehr unregelmäßig auf, und es bestand keine sichere Beziehung zwischen direktem Coombs-Test und Anämie. Die Ergebnisse zeigen, daß die subleukämische Anämie und die positiven serologischen Reaktionen nicht durch Autoantikörper bedingt waren. Sie lassen vielmehr annehmen, daß die Anämie durch eine Verbindung von Viruspartikeln mit den Erythrozyten hervorgerufen wird.

Résumé

Afin d'élucider si le décours atypique de la leucémie myéloïde, l'anémie subleucémique, une cause immunologique, le virus de la leucémie myéloïde été inoculé à des poussins. Ils ont été soumis à des examens hématologiques et sérologiques, y compris une épreuve de Coombs avec un sérum de lapin anti-poule. Trois groupes d'âges différents ont été examinés soit des poussins âgés de 9, 21 et 70 jours lors de l'inoculation du virus. Aucune réaction positive ne fut observée dans le groupe âgé de 70 jours. Parmi les poussins âgés de 9 et de 21 jours lors de l'inoculation, 50, respectivement 35%, présentèrent une épreuve de Coombs positive. Les réactions sérologiques eurent lieu irrégulièrement, aucune relation certaine n existant entre une épreuve de Coombs directe positive et l'anémie. Ces résultats indiquent que l'anémie subleucémique et les *réactions sérologiques positives n étaient pas dues à des anticorps.* Ils font au contraire penser que l'anémie est causée par une fixation de particules de virus aux érythrocytes.

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Proceedings of the New Zealand Symposium on Geographical Haematology

The papers from this international symposium, held in Dunedin in 1966, will be published in full as supplement to the December 1966 issue of the New Zealand Medical Journal. Copies (about 150 pages) will be supplied free of charge to those who attended the Symposium. Others interested may obtain copies from the Guest Editor Dr F W Gunz, Christchurch Hospital, Christchurch (New Zealand) by sending a remittance for Sterling £ 1 – – or US $ 2.50.

Experimental Biology and Medicine

In September 30, 1966 scientists from 10 different countries met in Valbella near Chur (Switzerland) for a conference on Experimental Biology and Medicine. The main theme discussed by these specialists in the fields of genetics, biochemistry biophysics, microbiology and virology as well as experimental medicine, was the control mechanisms of the differentiation and the de-differentiation of cells—the search for the chemical nature of matter which determines the development of the embryonic cells into muscle, nerve, cartilage or even cancer cells.

The conference, which was supported by NATO, German ministries and the Volkswagen Foundation, was initiated and organized by Professor Ernst Hadorn (Bonn) Professor F Zilliken (Marburg) and Dr W Wechsler (Cologne) The conference was held under the patronage of the publishing firm S. Karger AG (Basel) who will bring out the details report.

The results of the scientific discussions of the international conference were so stimulating and constructive that it was decided to hold a similar conference at the same place in tw years time to discuss further developments in research.

Transplantation Society

The First International Congress of the Transplantation Society will be held in Paris from June 27 to 30, 1967 Programme. Mechanisms of graft rejection methods of immuno-depression genetics of transplantation; transplantation antigens, organ transplantation; bone marrow transplantation cancer as homograft. Co-chairmen: Prof. Ag. J Dausset Prof. J Hamburger, Prof. G Mathé. Secretariat J Dausset Hôpital Saint Louis, Place du Dr Fournier Paris X (France).

This Congress will be preceded on June 26 by Colloquium on Organ Transplantation [Prof. J Hamburger, Hôpital Necker Paris 15e (France)].

International Society of Haematology

At the 11th Congress of the International Society of Haematology in Sidney from August 21–26, 1966, the amalgamation of the International Society the European Society and the Asian-Pacific Society of Haematology was approved by the Board of Councillors and the General Meeting, so that there is now only one International Society of Haematology consisting of three Divisions the Inter American, the European and the Asian-Pacific. It is intended that each Division can have and nominate up to three Vice-Presidents and one Secretary-General/Treasurer For the interim period up to the Congress in 1968 temporary ppointments were made by the Board of Councillors for the European Division as follows: Vice-Presidents Drs. J Bernard S. I de Vries and J F Wilkinson; Secretary-General and Treasurer Dr H. Braunsteiner. Acta Haematologica will now be the official organ of the European Division.

The next Congress of the International Society of Haematology will take place t the beginning of September 1968 in New York (President Prof. C. Moore; Secretary-General Dr P Reizenstein 449 East 68th Street, New York).

A Meeting of the European Division of the International Society of Haematology will be held in Athens from September 10–14 1967 under the presidency of Prof. A. Gouttas (see Acta haemat. 36. 417 1966)

IIIème Symposium international sur la recherche comparée dans les leucémies
Paris, 11 13 juillet 1967

Symposium organisé sous les auspices du *World Committee for Comparative Leukemia Research*. Comité d'organisation H. Dieter, M. Boiron A. Parodi.

Principaux sujets traités

11 juillet Interaction entre cellules et virus oncogènes RNA. *Progrès dans le domaine des virus leucémogènes.*

12 juillet Leucémies bovines, étiologie, *épidémiologie pathologie et diagnostic*

13 juillet Leucémies canines et félines. *Leucémies humaines.*

Pour tous renseignements et inscriptions, *s'adresser: Prof. A. Parodi École* Vétérinaire – Alfort – 94 Prof. M. Boiron, *Institut de Recherches sur les Leucémies*, Hopital Saint Louis, Paris Xe.

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Bearbeitet von G. Bo[illegible], Basel

(B) = Buchbesprechungen – Livres nouveaux – Book reviews

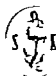
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Acta haemat. 38: 1–10 (1967)

Departments of Medicine (Head: Prof. F. Koller) and Dermatology (Head: Prof. R. Schuppli), University of Basle

Reversible Sideroblastic Anemia Caused by Chloramphenicol*

E. A. Beck, G. Ziegler, R. Schmid and H. Lüdin

Hazardous side-effects of chloramphenicol therapy primarily involve bone marrow function. The various patterns of reversible and irreversible depression of hematopoiesis caused by chloramphenicol have been reviewed in recent years (1, 2). The production of red cells appears to be most frequently influenced by chloramphenicol (3) and the depression of erythropoiesis may depend on the concentration of free chloramphenicol in the circulating blood (4, 5). Vacuolization of erythroid precursors has been demonstrated as a consequence of chloramphenicol toxicity (6, 7) and this alteration may be reversed by ingestion of phenylalanine (7). A decrease in the turnover of serum iron appears to be predictive of chloramphenicol toxicity (8, 9) but the mechanism of this effect remains to be elucidated.

A possible relationship between chloramphenicol toxicity and the appearance of abnormal sideroblasts in the bone marrow was not mentioned in recent reviews on acquired sideroblastic anemia (10) and on bone marrow toxicity of chloramphenicol (1, 2). Evidence was recently obtained that chloramphenicol may produce reversible sideroblastic anemia in certain individuals. The clinical picture of several patients with this abnormality will be reported in the present paper.

Materials and Methods

Case records and bone marrow smears of patients with one or several of the following diagnoses were reevaluated: bone marrow depression caused by chloramphenicol; pancytopenia of unknown origin; and sideroblastic anaemias. Only patients ad-

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* Dedicated to Prof. A. Werthemann on the occasion of his 70th birthday.

mitted between January 1 1963, and October 31 1966, were included in the study The control group consists of 120 consecutive bone marrow studies done in 1966.

Bone marrow smears were stained by the MAY-GRÜNWALD-GIEMSA technique. Iron stains were performed according to the procedure described by LEDER (11).

Evaluation of sideroblasts was similar to the one described by HALL and LOSOWSKY (12).

Heme precursors in red cells and urine were measured according to methods described by WALLOCK (13)

Case Reports

Case I (Fig 1) This 58-year-old man was admitted four times, from 1960 through 1963, with chronic obstructive bronchitis, cor pulmonale and *recurrent* bronchopneumonia. The infection was treated over prolonged periods of time with one gram of chloramphenicol per day Bone marrow studies were performed in 1960 and 1962 because of persistent mild thrombocytopenia. No abnormal sideroblasts were detectable while the patient was on chloramphenicol treatment. The platelet counts were not significantly influenced by chloramphenicol therapy and no other significant hematologic abnormalities occurred during administration of chloramphenicol. In 1963, the patient again received chloramphenicol, but the daily dose was increased to 2 g Thrombocytopenia was still present and progressive anemia was noted. Bone marrow studies were repeated at time when the total doses of chloramphenicol reached 150 g Pertinent findings were a hypercellularity of erythropoesis with moderate macrocytosis and a marked increase of coarsely granulated sideroblasts. A moderate increase in the pro-

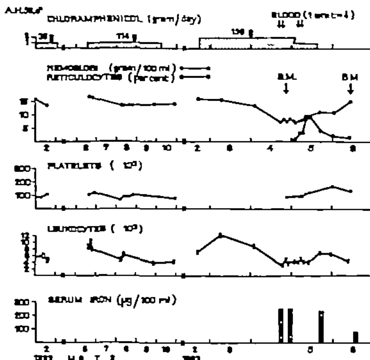


Fig 1

portions of both immature granulocyte precursors and megakaryocytes also was noted. The patient received 4 units of whole blood, and the chloramphenicol dose was reduced to 1 g per day. On this reduced dose reticulocyte counts increased from 0.9 to 9.1 %. After withdrawal of the drug hemoglobin values reached normal limits without further transfusions. Elevated serum iron levels were observed during chloramphenicol treatment, and normal serum iron value was obtained shortly after withdrawal of the drug. Bone marrow aspiration four weeks after withdrawal of chloramphenicol showed no abnormal sideroblasts.

Case 2 (Fig. 2). This 79-year-old male patient was admitted in August, 1966, for evaluation of normochromic anemia and weight loss. Examination revealed an enlarged prostate, chronic renal disease and congestive heart failure. The patient probably had intravascular hemolysis, and reticulocyte counts were constantly elevated. 2 g of chloramphenicol per day were administered because of urinary tract infection and first bone marrow aspiration was performed at time when total dose of 8 g of this drug had been given. Bone marrow examination revealed no abnormalities. Concomitantly with further administration of chloramphenicol reticulocyte counts decreased from 5.8 to 0.1% and mild decrease in hemoglobin values also was noted. Bone marrow examination after total dose of 38 g of chloramphenicol revealed marked increase in the proportion of coarsely granulated sideroblasts, immature erythroid and granulocyte precursors as well as diffuse lymphoid infiltration. Porphyrins in the red cells and urine, Coombs test, hemoglobin electrophoresis and glucose-6-phosphate dehydrogenase levels were normal. Chloramphenicol therapy was stopped. Shortly thereafter the reticulocyte counts rapidly increased. Hemoglobin values reached pretreatment levels without any transfusions. Bone marrow smears again were studied two weeks after withdrawal of chloramphenicol. The previous abnormalities, including the abnormal sideroblasts, had completely disappeared.

Case 3 (Fig. 3). This 63-year-old male patient was twice admitted in 1966 with asthma bronchiale, right ventricular failure and psoriasis. Chloramphenicol was administered on several occasions for treatment of bronchial infection. Hemoglobin concentrations and reticulocyte counts decreased following prolonged administration of

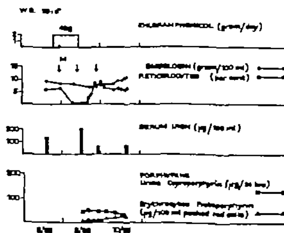


Fig. 2.

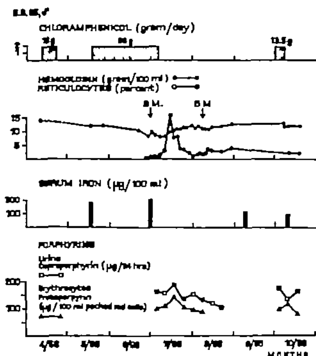


Fig 3.

2 g of chloramphenicol per day during May and June, 1966. Bone marrow studies were performed after administration of total dose of 84 g of chloramphenicol. Abnormal findings were erythroid hyperplasia with an increased proportion of immature normoblasts and striking increase in the proportion of coarsely granulated and 'ringed' sideroblasts. Serum iron levels were increased. Erythrocyte protoporphyrin was markedly elevated, and excretion of coproporphyrin in the urine also was increased, but other heme precursors were found to be normal. After withdrawal of chloramphenicol rapid increase in the reticulocyte counts from 0.1 to 16% was noted, and hemoglobin values slowly returned to normal levels. White cell and platelet counts had remained within normal limits. Bone marrow studies were repeated one month after withdrawal of chloramphenicol and complete disappearance of the previously observed abnormalities was noted. Another short course of chloramphenicol therapy did not produce any significant changes in blood counts.

Results

Control group This group includes a variety of hematologic disorders including anemia secondary to chronic renal disease, infection or vitamin B_{12} deficiency as well as normal bone marrows. Iron stains were restudied with respect to the presence of sideroblasts. Smears with no detectable deposition of hemosiderin were not included, and bone marrow studies on patients with abnormal sideroblasts and/or chloramphenicol toxicity are reported below

Table I
Incidence and probable cause of sideroblastic anemia.

Type	Probable cause	Number of patients
Acquired, chronic	unknown	8
Acquired, reversible	chloramphenicol	18
	vit. B_{12} deficiency	1
	lead poisoning	1

The sideroblast count usually was zero, and the proportion of discretely granulated sideroblasts did not exceed 5%. Sixteen patients with a normal sideroblast score had received chloramphenicol at the time of the bone marrow study the daily dose being 2 g in most instances.

Patients with abnormal sideroblasts During the period of the study bone marrow smears obtained from 28 patients contained an elevated proportion of abnormal sideroblasts (Table I) Quantitative evaluation revealed that 10 to 97, of the nucleated red cell precursors contained coarse iron granules. Eight patients of this group had refractory anemia that did not respond to elevated doses of pyridoxine, folate and vitamine B_{12}. Reversible sideroblastic anemia was observed *in one patient with pernicious anemia before treatment* and in one patient with chronic lead poisoning An increased proportion of abnormal sideroblasts was observed in 18 patients concomitantly with or shortly after chloramphenicol therapy The score of anormal sideroblasts was not related to the total amount of chloramphenicol administered at the time of bone marrow aspiration. Again, most of these patients received 2 g of chloramphenicol per day Eleven patients developed reversible anemia, one patient had reversible thrombocytopenia after total doses of 20 and 14 g of chloramphenicol respectively two patients developed anemia as well as thrombocytopenia, and reversible pancytopenia was seen in two patients (Table II) No detectable changes in blood counts were observed in two patients at a time when a marked increase in the proportion of sideroblasts was found on *bone marrow preparations*. The disappearance of sideroblasts after withdrawal of chloramphenicol was documented by repeated bone marrow studies in 5 patients including the 3 previously described cases. The abnormal blood counts rapidly returned to pre-treatment values after withdrawal of chloramphenicol in the remaining patients.

Table II

Incidence of hematologic alterations observed during and shortly after chloramphenicol therapy

Blood		Bone marrow sideroblast.	vac.	sideroblast. + vac.
Anemia	18	7	2	4
Thrombocytopenia	1	1	–	–
Anemia + thrombocytopenia	3	–	(1)	2
Pancytopenia	4	2	1	–
Suppression of response to iron therapy	1	–	–	–
No change	20*	1		1

Twice observed in same patient after small dose.
Including control group 1966.

Patients with bone marrow depression caused by chloramphenicol. Table II summarizes the hematologic observations in patients who had received chloramphenicol and were referred to the hematology service for detailed studies while receiving chloramphenicol or shortly after withdrawal of chloramphenicol. Reversible anemia was most frequently seen, and depression of platelet and white cell counts were less often encountered. Vacuolization of red cell precursors and/or abnormal sideroblasts were detected on bone marrow smears obtained from a majority of these patients One woman with documented iron deficiency did not respond to iron treatment while on chloramphenicol therapy but promptly recovered from the anemia after withdrawal of chloramphenicol. In another patient with combined anemia and thrombocytopenia produced by chloramphenicol both abnormal sideroblasts and vacuolization of red cell precursors were demonstrable one day after withdrawal of the drug Five days later the sideroblasts had disappeared but discrete vacuolization was still seen, and 10 days later both changes had disappeared

The data obtained from studies of those patients who were studied *during* administration of chloramphenicol are summarized in Table III An increased proportion of abnormal sideroblasts, but no vacuolization of red cell precursors, was found on bone marrow smears obtained from 9 patients. This type of abnormality was most frequently associated with pure reversible anemia and was more often found in men than in women. Vacuolization of red cell precursors and abnormal sideroblasts were concomitant findings in 5 patients, and vacuolization alone was present in red cell

Table III

Incidence of hematologic alterations observed during administration of chloramphenicol.

Patient (age, sex)	Siderobl.	Vac.	Anemia	Anemia + thrombocytop.	Pancytop.
T R., 71 M.	+				
U S., 69, M.	+		+		
J H., 54 M.	+		+		
E B., 66, M.	+		+		
W S., 79 M.	+		+		
F E., 67 M.	+		+		
G B., 76, F	+		+		
W U 51 M.	+		+		
H. D 70, M.	+	+			
E S., 27 M.	+	+	+		
O. G., 56, M.	+	+	+		
R. Z., 62, F	+	+	+		
J T 46, M.	+	+		+	
M. H., 55 F		+		+	
L. B., 67 F	+				+
E. Q., 61 F		+			+
Total 16	14	7	10	2	2
M: 11	11	4	8	1	–
F: 5	3	3	2	1	2

precursors obtained from two individuals. Other abnormal findings on these bone marrow preparations were an increase in the proportion of immature precursors of red cells, granulocytes and megakaryocytes as well as diffuse infiltration of lymphoid and plasma cells.

Evaluation of renal and hepatic function in patients with symptoms of chloramphenicol toxicity revealed that 3 patients had a marked decrease in the rate of glomerular filtration, 2 patients had evidence of liver disease, and 2 patients had combined renal and hepatic disturbances. However no such predisposing factors were demonstrable in the remaining patients of this group

Discussion

Reevaluation of records of patients with sideroblastic anemia demonstrates that acquired sideroblastic anemia frequently was associated with chloramphenicol medication. An increase in the proportion of sideroblasts also was observed in patients with symptoms of bone marrow depression by chloramphenicol. Disappearance of the abnormal sideroblasts was demonstrated in 5 patients shortly after withdrawal of chloramphenicol It appears therefore that

Table II
Incidence of hematologic alterations observed during and shortly after chloramphenicol therapy

Blood		Bone marrow siderobl.	vac.	siderobl. + vac.
Anemia	18	7	2	4
Thrombocytopenia	1	1	–	
Anemia + thrombocytopenia	3	–	(1)	2
Pancytopenia	4	2	1	–
Suppression of response to iron therapy	1	–	–	–
No change	20*	1	–	1

Twice observed in same patient after small dose.
Including control group 1966.

Patients with bone marrow depression caused by chloramphenicol. Table II summarizes the hematologic observations in patients who had received chloramphenicol and were referred to the hematology service for detailed studies while receiving chloramphenicol or shortly after withdrawal of chloramphenicol. Reversible anemia was most frequently seen, and depression of platelet and white cell counts were less often encountered. Vacuolization of red cell precursors and/or abnormal sideroblasts were detected on bone marrow smears obtained from a majority of these patients. One woman with documented iron deficiency did not respond to iron treatment while on chloramphenicol therapy but promptly recovered from the anemia after withdrawal of chloramphenicol. In another patient with combined anemia and thrombocytopenia produced by chloramphenicol both abnormal sideroblasts and vacuolization of red cell precursors were demonstrable one day after withdrawal of the drug Five days later the sideroblasts had disappeared but discrete vacuolization was still seen, and 10 days later both changes had disappeared

The data obtained from studies of those patients who were studied *during* administration of chloramphenicol are summarized in Table III An increased proportion of abnormal sideroblasts, but no vacuolization of red cell precursors was found on bone marrow smears obtained from 9 patients. This type of abnormality was most frequently associated with pure reversible anemia and was more often found in men than in women Vacuolization of red cell precursors and abnormal sideroblasts were concomitant findings in 5 patients, and vacuolization alone was present in red cell

to recognize the individual susceptibility to chloramphenicol and the need of careful hematologic evaluation of every patient on chloramphenicol therapy

Summary

During four-year period, abnormal sideroblasts were demonstrated on bone marrow smears obtained from 18 patients concomitantly with or shortly after chloramphenicol therapy The relation between chloramphenicol treatment and formation of abnormal sideroblasts is illustrated by 3 case reports. Sideroblastic anemia ppeared during chloramphenicol therapy and disappeared upon withdrawal of the drug in these patients. However abnormal sideroblasts were not demonstrable in every instance of reversible bone marrow depression by chloramphenicol. The following additional findings may be associated with the development of sideroblastic anemia caused by chloramphenicol thrombocytopenia or reversible pancytopenia; decreasing reticulocyte counts; increased serum iron levels; increase in erythrocyte protoporphyrin and in the excretion of coproporphyrin in the urine. Irreversible bone marrow suppression by chloramphenicol was not observed during the period of the study

Zusammenfassung

Während einer Untersuchungsdauer von vier Jahren wurden 18 Fälle von sideroachrestischer Störung der Erythropoese unter oder kurz nach Behandlung mit Chloramphenicol beobachtet. Die Beziehung zwischen der Therapie mit Chloramphenicol und der sideroachrestischen Störung wird anhand von drei Fällen illustriert. Die sideroachrestische Anämie trat bei diesen Fällen während der Chloramphenicol-Behandlung auf und verschwand nach Absetzen des Antibiotikums. Im Zusammenhang mit einer durch Chloramphenicol bedingten sideroachrestischen Störung können die folgenden zusätzlichen Befunde erhoben werden eine Thrombopenie oder reversible Panzytopenie, erniedrigte Retikulozytenwerte, ein erhöhtes Serumeisen, sowie erhöhte Erythrozytenporphyrin- und Urinkoproporphyrinwerte. Während der Dauer dieser Untersuchung wurde kein Fall von irreversibler durch Chloramphenicol verursachter Knochenmarksaplasie beobachtet.

Résumé

En l'espace de quatre ans, 18 cas de trouble sidéro-achrestique de l'érythropoïèse ont été observés pendant ou peu après un traitement au chloramphénicol. Les relations existant entre l'administration de chloramphénicol et le trouble sidéro-achrestique qui peut en résulter sont illustrées par trois cas. L'anémie sidéro-achrestique est apparue dans ces trois cas durant le traitement chloramphénicol et disparu près sa cessation. Les troubles supplémentaires suivant peuvent accompagner le trouble sidéro-achrestique thrombopénie ou pancytopénie réversible diminution du nombre des réticulocytes, taux augmenté de fer sérique, de porphyrine érythrocytaire et de coproporphyrine urinaire. Pendant la durée de ces recherches, aucun cas d' plasie totale et irréversible de la moelle osseuse ne fut observé.

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Studies on Chloramphenicol Induced Haemolysis in vitro and Survival of Chloramphenicol Treated Cells in vivo

P. C. Khanduja and K. N. Agarwal

Chloramphenicol is well known to induce aplastic anaemia, thrombocytopoenia and leukaemia. These manifestations are most likely due to a abnormal trigger to the stem cell in the bone marrow. The occurrence of haemoglobinuria in subjects receiving chloramphenicol in therapeutic doses seems to be due to either red cell susceptibility i.e. deficiency of Glucose 6-phosphate dehydrogenase (3, 5) or that chloramphenicol is capable of causing morphological and metabolic changes in the human red cell. In this study some of the *in vitro* changes induced by chloramphenicol, and survival of such damaged cells *in vivo* is discussed.

Material and Methods

Chloramphenicol pure substance (Chloromycetin) was obtained from Parke Davis & Co. (USA). It was dissolved in methanol (50 mg in 0.5 ml of methanol) and desired solutions were finally made in phosphate buffer (Sorensen phosphate buffer pH 7.4 at 20 °C. Cp. Documenta Geigy Scientific tables page 314) before addition to the red cell suspension. Control buffer solution with equal volume of methanol was also prepared in each experiment.

Blood samples were taken from healthy donors in heparinized tubes containing 100 I. U. of heparin for 20 ml of blood, plasma and buffy coat removed after centrifugation at 600 × g for 5 min. The cells were washed once with the phosphate buffer and finally suspended in buffer. Experiments were made within 2 h of collection of blood.

Prednisolone-hemisuccinate was obtained from Organon, Oss. (Holland); solutions were prepared in the buffer.

Chloramphenicol induced haemolysis. The various concentrations of chloramphenicol were incubated with the red cell suspensions at 37 °C in water bath for 3 to 4 h. The suspensions were mixed off and on. In each experiment parallel tubes were put containing control buffer solutions. The samples were centrifuged after the completion of experiment and percentage of haemolysis calculated as described by Agarwal and Garby (2).

Effect on changes in red cell volume. The non-haemolytic concentrations of chloramphenicol were incubated with red cells and the haematocrit determined at various intervals,

after centrifugation for 5 min t about 11000 × g in Adams Readocrit, (Micro-haematocrit Centrifuge) Inc. N.Y (USA)

The osmotic fragility studies were made with various concentrations of chloramphenicol by the method of SJOLIN (1954) The osmotic fragility of chloramphenicol damaged cells after resuspension in the whole blood of the same individual was also studied.

Effect on red cell morphology. The red cells were incubated with chloramphenicol as above and changes in the cell morphology were studied at different intervals by using the Giemsa stain or incubating the cells in brilliant cresyl blue solution.

Potassium loss. The red cell suspensions containing control buffer and chloramphenicol were incubated at 37 °C, aliquots were taken t different intervals to determine the potassium content in the supernatants by flame photometry

Methaemoglobin formation. The red cell suspension was incubated with chloramphenicol and methaemoglobin formation was measured by the EVELYN MALLOY method (1938)

Acid serum test was done according to HAM (7)

Inhibition of haemolysis. Experiments were designed to incubate red cells with buffer prednisolone, Mg++ and sucrose solutions for 15 min prior to addition of chloramphenicol.

Survival of chloramphenicol treated cells in vivo Eight ml of blood were taken in heparinized syringe, transferred into sterile rubber capped test tube and centrifuged for 5 min at 600 × g The plasma and buffy coat were removed and the cells washed with isotonic saline. 2.0 ml of packed red cells were transferred to sterile tube. To this appropriate amounts of chloramphenicol solution or identical volumes of methanol + phosphate buffer (control cases) were added, the suspensions were incubated for 30 min t the room temperature. Thereafter 40 μc of Cr^{51} (Atomic Energy Establishment Trombay) was added and incubated for 30 min. The cells were washed twice with isotonic saline and reinjected. Blood samples were collected from the opposite arm at different intervals. The samples were centrifuged and plasma separated for counting. The haemolysed blood samples and plasma were counted in well type scintillation counter

Results

The control buffer did not cause haemolysis *per se*

Chloramphenicol induced haemolysis The relationship between the concentrations of chloramphenicol and haemolysis studied is shown in Fig 1 These experiments show that the degree of haemolysis is related to the increasing concentrations of chloramphenicol and also the similar concentrations in different bloods induce variable haemolysis.

Effect on changes in red cell volume The change in red cell volume on incubation with 66 μmoles/ml RBC of chloramphenicol caused increase in red cell volume while there was no significant change in the control (Fig 2)

Effect on osmotic fragility The incubation of washed buffered cells in various concentrations of chloramphenicol produced dose related increase in osmotic fragility with a leftward shift of the curve (Fig 3) In Fig 4 is shown the effect of prednisolone on

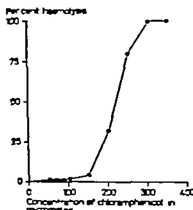


Fig 1 Haemolysis of red cells in relation to concentration of chloramphenicol. Incubation time 4 h.

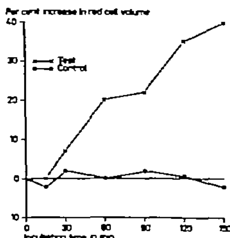


Fig 2. Effect of chloramphenicol (66 µmoles/ml RBC) on erythrocyte volume.

chloramphenicol induced changes, it shows that prednisolone protects against the osmotic fragility changes. The chloramphenicol treated cells after incubation with normal plasma at 37 C for 30 min showed fragility curve similar to control.

Effect on red cell morphology The slides stained with Giemsa and those made after incubation with brilliant cresyl blue showed some degree of cellular enlargement and spherocytosis. No Heinz body formation was observed.

Potassium loss. The results show that chloramphenicol induces potassium loss (Fig 5)

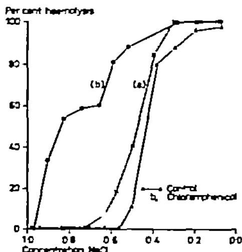


Fig 3 Effect of chloramphenicol on erythrocyte osmotic fragility in relation to concentration of chloramphenicol. *( )* 77 μmoles/ml of RBC. *(b)* 220 μmoles/ml of RBC.

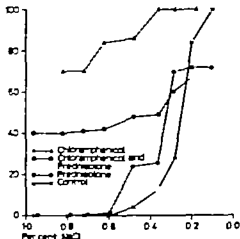


Fig 4 Effect of prednisolone on chloramphenicol induced osmotic fragility changes. (Prednisolone concentration 181 μmoles/ml of RBC. Chloramphenicol concentration 254 μmoles/ml of RBC)

Methaemoglobin formation. Chloramphenicol induces intracellular methaemoglobin formation (fig 6)

Acidified serum test. No haemolysis is seen after incubation with chloramphenicol

Inhibition of haemolysis. The data on inhibitory effect of prednisolone, Mg++ and sucrose is given in Table I and II. It is possible to inhibit the haemolysis completely with increasing doses of these substances.

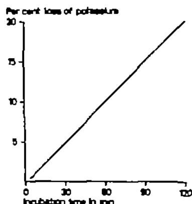


Fig 5 Erythrocyte potassium loss during incubation with chloramphenicol (55 μmoles per ml of RBC)

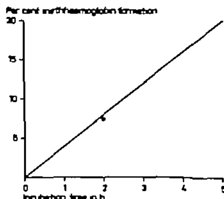


Fig 6 Methaemoglobin formation during incubation with chloramphenicol (52 μmoles/ml of RBC)

Survival of chloramphenicol treated cells in vivo The control and chloramphenicol treated red cells (incubated with doses of 130 200 and 260 μmoles/ml of RBC for 30 min) showed no change *in vivo* after reinjection in circulation during 24 hours period.

Discussion

It is generally recognised that the administration of certain drugs cause acute haemolytic anaemia. We have had occasion to observe two cases of chloramphenicol induced haemoglobinuria (5) The patients showed deficiency of the red cell enzyme glucose 6-phosphate dehydrogenase (G6PD) In this study some data

Table I
Inhibition of chloramphenicol induced haemolysis by prednisolone.

Experimental data	Conc. of prednisolone in μmoles/ml RBC	Percent haemolysis
V = 4.7 ml	0	4.20
Cm = 190 μmoles/ml RBC	0	4.00
T = 3 h	23.0	2.20
	46.0	1.10
	69.0	1.00
	92.0	0.51
	138.0	0.30
	184.0	0.00
V = 4.2 ml	0	39.7
Cm = 102 μmoles/ml RBC	180.0	25.8
T = 4 h	360.0	11.3
	540.0	10.7
	900.0	4.3
	1080.0	2.1
	1440.0	1.6
	1800.0	0.0

Table II
Inhibition of chloramphenicol induced haemolysis by Mg^{++} and sucrose.

Experimental data	Conc. of Mg^{++} in mmoles/ml of RBC	Percent haemolysis
V = 5.7 ml	0.00	82.2
Cm = 510 μmoles/ml of RBC	0.00	84.4
T = 3 h	0.17	76.6
	0.35	65.5
	0.70	64.4
	1.39	63.3
	2.09	62.3
	sucrose in mmoles/ml of RBC	
	0.00	65.5
	0.00	63.3
	0.97	11.1
	1.94	9.4
	3.98	8.8
	7.96	5.0
	11.94	3.8

V Volume, *Cm* Chloramphenicol, *T* Time, *h* hour

regarding chloramphenicol induced haemolysis *in vitro* and survival of such damaged human erythrocytes *in vivo* has been discussed.

The data indicate that lysis of erythocytes by chloramphenicol *in vitro* causes a failure to maintain the cation gradient, followed by cellular swelling and osmotic lysis. Chloramphenicol also induces formation of intracellular methaemoglobin. These findings are similar in many ways to the haemolysis induced by sulfhydryl inhibitors (4) especially with the mechanism of NEM (N-ethylmalimide) induced methaemoglobinaemia. In contrast, chloramphenicol did not cause Heinz body formation *in vitro* JACOB and JANDL (4) and AGARWAL (1) demonstrated that erythrocytes damaged by these sulfhydryl inhibitors (non haemolytic doses) *in vitro* disappear rapidly from the circulation on reinjection. Contrary to this experiments performed after incubation *in vitro* with chloramphenicol did not show any significant change in red cell survival *against the control. It is well known that chloramphenicol does not* block the sulfhydryl enzymes (6) and our observations also support that red cell changes induced by chloramphenicol are not due to binding on the sulfhydryl groups. Furthermore, the chloramphenicol induced red cell damage is reversible on incubation with plasma *in vitro* and there is lesser degree of haemolysis on addition of chloramphenicol to whole blood. Thus the normal *in vivo* survival may be due to plasma or its components having the capacity to normalise the damaged cells.

The *in vitro* haemolysis and the osmotic changes could be prevented by prior incubation with prednisolone. The mechanism of action of prednisolone may be due to the protection against the osmotic change rather than the binding to or closely to sulhfydryl groups (2) Similarly sucrose and Mg++ were capable of preventing the chloramphenicol induced haemolysis.

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Summary

The incubation of chloramphenicol with human red cells *in vitro* induced cellular swelling, loss of potassium, osmotic changes, formation of methaemoglobin and finally haemolysis. These changes could be protected *in vitro* by prior incubation with prednisolone, Mg++ sucrose and plasma. The chloramphenicol damaged cells after tagging with Cr^{51} when reinjected showed normal survival *in vivo*.

Zusammenfassung

Die Inkubation menschlicher Erythrocyten mit Chloramphenicol *in vitro* führt zu Zellschwellung, Kaliumverlust, osmotischen Veränderungen, Methämoglobinbildung und schließlich zur Hämolyse. Diese Veränderungen lassen sich *in vitro* verhindern durch vorhergehende Inkubation mit Prednisolon, Mg++ Saccharose und Plasma. Nach Markierung mit Cr^{51} zeigen die durch Chloramphenicol geschädigten Erythrocyten bei Reinjektion *in vivo* eine normale Lebensdauer

Résumé

L'incubation *in vitro* d'erythrocytes humains avec du chloramphénicol provoque une enflure, une perte de potasse et des altérations osmotiques cellulaires ainsi que la formation de methémoglobine et enfin une hémolyse. Ces altérations purent être évitées *in vitro* par l'incubation préalable avec de la prédnisolone, du Mg++ de la saccharose ou d plasma. Le temps de survie *in vivo* d'érythrocytes altérés par le chloramphénicol, puis radio-marqués au Cr^{51} et réinjectés, était normal.

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The Proliferation of Infectious Mononucleosis Lymphocytes in vitro

E. H. Cooper, A. J Hale and J D Milton

Studies of the proliferation of leucocytes in various circumstances may be expected to yield information about mechanisms of control of leucocyte production. In the course of its mitotic cycle the cell passes through four distinct stages G_1, a stage in which the DNA content is diploid (20) and DNA synthesis has not commenced S a stage of replication of the DNA, when the DNA content increases from 2c to 4c (tetraploid) G_2, a period in which the cell nucleus has a 4c DNA content and is awaiting the onset of mitosis finally mitosis (M) It is convenient to consider that these stages in the division cycle represent clear cut compartments. The relative distribution of cells amongst the compartments provides some information about the proliferative activity of the cell population far more information can be obtained about proliferation rates when the passage of cells from one compartment to another in the mitotic cycle is studied (12)

It is now well established that the atypical mononuclear lymphocytes that are present in the peripheral blood during the active phase of infectious mononucleosis are proliferating A proportion of the atypical lymphocytes in the peripheral blood during the active phase of infectious mononucleosis are synthesising DNA (2, 7 8, 9 10 14 19) Although these DNA synthesising cells may represent up to 15% of the total peripheral blood leucocytes, mitotic figures are rarely seen in routine blood films and only few are found on examination of buffy coat films (7) Furthermore, it has been shown that when the cells are put into culture only 50% of the DNA synthesising cells are capable of going on to division (4)

Zusammenfassung

Die Inkubation menschlicher Erythrocyten mit Chloramphenicol *in vitro* führt zu Zellschwellung, Kaliumverlust, osmotischen Veränderungen, Methämoglobinbildung und schließlich zur Hämolyse. Diese Veränderungen lassen sich *in vitro* verhindern durch vorhergehende Inkubation mit Prednisolon, Mg++ Saccharose und Plasma. Nach Markierung mit Cr^{51} zeigen die durch Chloramphenicol geschädigten Erythrocyten bei Reinjektion *in vivo* eine normale Lebensdauer

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This paper is an account of studies of atypical lymphocytes in which a combination of autoradiographic and microdensitometric methods have been used to investigate the rate of their movements accross the compartments of interphase *in vitro*

Materials and Methods

Leucocyte preparations. Leucocytes were obtained from 20 patients in the acute phase of infectious mononucleosis, who were febrile and had enlarged lymph nodes at the time the blood was taken. Nineteen of these patients had positive Paul and Bunnell hetrophile antibody reactions, the titres ranging from 1:244 to 1 1768. The patients were aged between 12 and 17 and peripheral blood leucocyte counts between 4,000–23,000 per mm³ One patient, a boy aged 12, had a coincidental orbital cellulitis and was under treatment with penicillin at the time of taking the blood samples. 10–15 ml of venous blood was drawn into a dry syringe. Approximately 2 ml were put into a sequestrene bottle for routine haematological analyses and the remainder was put into a bottle containing heparin. The heparinized blood was left to stand at 37° C for 20–30 min after this time the leucocyte containing plasma was removed and mixed 1 to 1 v/v with tissue culture medium TC 199 (Glaxo) The incorporation of the radioactive precursors into the cell was studied with the cells in this incubation medium.

Phytohaemagglutinin cultures were made with samples of venous blood taken from healthy donors the cultures were set up using established methods (17). Phytohaemagglutinin (PHA) was obtained from the Wellcome Research Laboratories.

Radioactive precursors: Tritiated thymidine (^{3}H TdR), specific activity 1.9 C/m Mole and ^{14}C-thymidine (^{14}C-TdR) specific activity 37.9 mc/mM, were used at concentrations of 1 or 2 μC/ml. These isotopes were obtained from the Radiochemical Centre, Amersham, Bucks. Tritiated deoxycytidine-5 -monophosphate (^{3}H-dCMP) specific activity 1.0 C/m Mole (Schwarz Bioresearch Inc., Orangeberg, New York) was used at a concentration of 1 or 2μ C/ml. Both these molecules are specifically incorporated into DNA. It has been shown that human myeloid leucocytes can degrade thymidine to thymine and dihydrothymine *in vitro* (11 15, 16) these degradation products are not used by the cells for DNA synthesis. This degradation of thymidine could have an effect on the labelling pattern of the cells in cultures incubated with the precursor for 4–5 h. On the other hand, it has been demonstrated that ^{3}H-dCMP is source for DNA cytosine and thymine, and because of its particular metabolism acts as a source of DNA label for a longer period than thymidine (11)

Measurement of the rate of accumulation of labelled metaphases and the number of cells in DNA synthesis. The radioactive precursor was added to the suspension at the beginning of the experiment and aliquot of the cells withdrawn after various intervals of time, the incubations being made at 37° C. At the end of the incubation the cells were washed with Hanks' solution, fixed with methanol and acetic acid (3 1 v/v) and these cells dropped on chilled slides. The majority of these preparations were stained by the Feulgen method prior to coating with autoradiographic emulsion. Others were stained through the developed and fixed films using MacNeal' tetrachrome stain at pH 6.5. The preparations were examined after exposure and development of the autoradiographs and the percentage of interphase cells and metaphases labelled by the radioactive precursor determined. Metaphase arrest, when required, was effected by the addition of colchicine to the suspension of cells at the beginning of the experiment to concentration of 1×10^{-4} M.

Double labelling experiments. The cultures were labelled with 1 μC/ml ^{3}H-TdR for 30 min, washed with TC 199 + plasma at 37° and resuspended in this medium. The

incubations were then continued for further 4 h, and finally ^{14}C-TdR 1 μC/ml was added for either 15 or 30 min. The cells were then spread on slides, fixed with methanol and stained by the FEULGEN method at pH 1.6 and autoradiographs prepared. Photographic maps were made of the slides, the position of the labelled cells recorded and the type of the labelling, whether ^{3}H, ^{14}C or ^{14}C and ^{3}H noted (20) The autoradiographic grains were then removed and the DNA content of these labelled cells measured by microdensitometry using the methods as previously described (1 12)

Results

The relation between the number of cells synthesising DNA to the number of atypical lymphocytes per mm³ in the peripheral blood is shown in Fig 1 The relation between the number of atypical lymphocytes synthesising DNA to the number of metaphases per mm³ is shown in Fig 2 The number of metaphases was calculated from the frequency of metaphases per 1 000 labelled cells and the percentage of total leucocytes that were labelled with the radioactive precursor after 30 min incubation. Hence, these figures for the frequency of metaphases were derived from the survey of 10,000–50 000 leucocytes. Metaphases were seen in all the concentrated leucocyte preparations from the patients with infectious mononucleosis, but they were only rarely seen when routine blood films from patients were examined for differential counting and diagnostic assessment, as has been noted by others (7) The discrepancy was due to the very much larger sample of the population of leucocytes examined in the concentrated leucocyte preparation. An unusually high frequency of metaphases was found in the patient with the intercurrent infection.

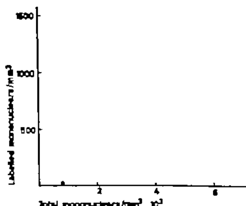


Fig 1 Relation between the number of atypical lymphocytes synthesising DNA and the total number of atypical lymphocytes per mm³ of peripheral blood.

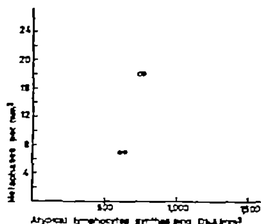


Fig 2. Relation between the number of atypical lymphocytes synthesizing DNA to the *number of metaphases per mm³ of peripheral blood (0 = Patient with orbital cellulitis)*

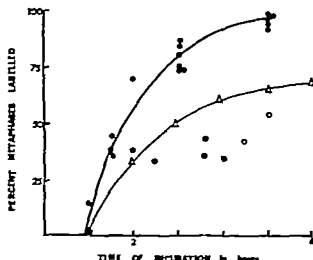


Fig 3. Rate of production of labelled metaphase figures in suspensions of infectious mononucleosis leucocytes incubated with ³H-dCMP ○ cultures blocked with colchicine. △ predicted percentage labelling in colchicine blocked cultures, derived from equation (1) ● unblocked cultures.

When samples of cells are incubated with DNA precursors the rates of increase of labelled metaphases can be measured Fig 3 shows the plot of this accumulation. The figure also shows the calculated and observed effects of a colchicine blockade on this accumulation of labelled metaphases. Fig 4 shows comparable data to that in Fig 3 derived from experiments on normal lympho-

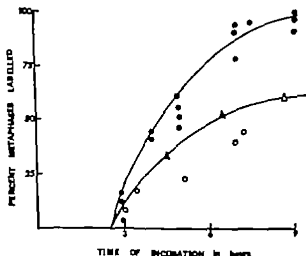


Fig 4 Rate of production of labelled metaphases in PHA culture incubated with ^{3}H-dCMP ○ cultures blocked with colchicine. △ predicted percentage labelling in colchicine blocked cultures, derived from equation (1) ● unblocked cultures.

cytes stimulated by PHA. It will be noticed that in Fig 3 and 4 the experimental results for the percentage of colchicine blocked metaphases is lower than that derived from theory (see below) This may be due to a possible hold-up of the movement of cells from prophase to metaphase due to the action of colchicine. Information about the time spent in G_2 and M can be obtained from these plots (13) The 50% intercept on this curve is a measure of the mean duration of $G_2 + \frac{1}{2}$ M. Thus we have found that $G_2 + \frac{1}{2} M = 1\frac{3}{4}$ h for the infectious mononucleosis cells. Similarly by counting the number of metaphases in the sample before and after adding colchicine one can calculate the duration of mitosis (6) (Table I) Likewise, the number of cells entering M each hour can be calculated. Thus the mean G_2 for the infectious mononucleosis cell *in vitro* is 1 h. The addition of colchicine to the cells caused a slight reduction in the percentage of labelled cells after 240 minutes incubation with ^{3}H-dCMP In one experiment 9.8% of the cells were labelled in the presence of colchicine and 10.1 % without colchicine in a second experiment these results were 11.2% and 11 7% respectively A steady increase in the percentage of labelled cells was observed to occur with increasing duration of incubation of the cells with ^{3}H-dCMP Table II shows this rise in absolute terms and also the calculated flux statistics for these

Table I
Effect of colchicine block on accumulation of mitotic figures.

Experiment No.	Initial M/mm³	Duration of incubation in h	Final M/mm³	K in M	Duration of M in h
169	7.1	2.0	15	4.0/h	0.95
171	15.8	3.5	27	3.2/h	2.1
187	8.3	3.5	30.5	6.3/h	0.93
186	7.5	3.5	21	3.9/h	1.25

Table II
Flux statistics of infectious mononucleosis cells *in vitro*.

Experiment	Total number of labelled cells per mm³ Duration of incubation in minutes							Flux cells/mm³ h		Ratio
	30	60	90	120	180	240	300	K in S	K in M*	K in S / K in M
108	1162		1344		1596		2058	194	4.0	48.5
153		1250			1400		1700	115	3.2	22
186	1036		1190			1414		108	3.4 (3.9)	32
187	910		1008		1344	1404		141	4.2 (6.5)	33
143	730			930		1430		200	(4.0)	50

Rate of alteration in the total numbers of labelled cells per mm³ blood when incubated in the presence of ³H-dCMP

* Values in brackets were derived from the increase of percentage of labelled metaphases with time of incubation with colchicine. The remaining values were obtained without the use of colchicine.

samples. The number of cells passing any point in or between phases in the cell cycle in unit time is the flux. $K_{in\,S}$ and $K_{in\,M}$ are the fluxes into S and M respectively

Maintaining the infectious mononucleosis cells in culture for 5 h did not alter their mitotic frequency (Table III) It was observed that there was a very considerable difference in the number of metaphases per 1 000 DNA synthesising cells in fresh blood samples from patients compared to this ratio in PHA cultures after 72 h culture. This was, however less marked when the PHA cultures were examined after 48 h growth (Table IV)

It was apparent that the infectious mononucleosis cells *in vitro* have an abnormally high rate of flux of cells into S compared to that into M, which, if the population had been in a steady state of

Table III

Effect of incubation on the frequency of mitotic figures in infectious mononucleosis.

Patient No.	Mitotic figures per 1,000 DNA synthesizing cells	
	30 min	5 h
1	7	7.5
2	11	7 6
7	11	8.0
8	6	5.0
10	12	14.0
12	20	23.0

Table IV

Relation of metaphase figures to DNA synthesizing cells.

Metaphases per 1,000 cells synthesizing DNA								
		Individual observations						Average
Infectious		7	11	5	8	12	4	
Mononucleosis		4	11	6	9	5	13	9.8
		11	12	11	20	5	18	
Phytohaemagglutinin								
cultures	72 h	48	40	30	47			42.0
		29	41	56	46	43		
	48 h	9	21	21	15			16.5
30 min incubation with ^{3}H-TdR								

proliferation, should have been approximately equal to one another or at the most not differing by a factor of greater than two. To obtain more evidence about the nature of the DNA synthesizing cells and their transit through S and G_2, a series of experiments using a combination of autoradiography and measurement of the DNA content of the individual nuclei by microdensitometry were made.

Fig 5 shows (a) the distribution of the DNA contents of the nuclei of cells that were synthesizing DNA as defined by autoradiographic labelling after a 30 minute exposure to ^{3}H TdR, (b) the lower histogram shows the change in the distribution pattern when cells had been continuously in contact with ^{3}H TdR for 3 h. In a normal proliferating population of cells this 5-hour sample would be expected to differ from the 30 minute sample by showing a

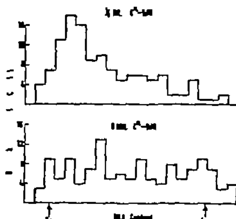


Fig. 5. Histograms of the distribution of amount of Feulgen staining (= DNA content) in the nuclei of infectious mononucleosis cells labelled with ¹⁴C-TdR. (a) after 30 min incubation with ¹⁴C-TdR. (b) after 5 h incubation, ¹⁴C-TdR added at the beginning of the experiment and after 4.5 h incubation.

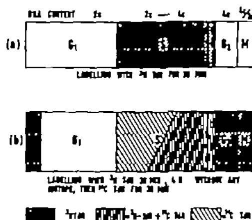


Fig. 6. Theoretical relation between expected labelling pattern and DNA content of cells. (a) situation after 30 min incubation with ³H TdR. Note that few labelled cells will have left the S compartment and moved into G_2. (b) distribution of labelling to be expected when the population of cells in the upper figure were incubated for 4 h without any isotope in the medium and then ¹⁴C thymidine was added for 30 min.

small but detectable build up of cells at the 4c mode (labelled cells in G_2) and a slight increase at the 2c mode (labelled G_1 cells arising by division of labelled cells) As the results obtained differed considerably from those expected for a normal proliferating cell population, double labelling experiments were made to analyse the nature of the disturbance of the normal pattern of cell kinetics that is a feature of these cells in culture.

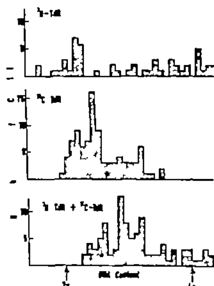


Fig 7 The relation between the labelling pattern and the DNA content of infectious mononucleosis cells that had been incubated with ^{3}H TdR for 30 min washed, incubated for 4 h and then incubated with ^{14}C-TdR for 30 min.



Fig 8. The relation between the labelling pattern and the DNA content of infectious mononucleosis cells that had been incubated with ^{3}H-TdR for 30 min, washed, incubated for 4 h, and then incubated with ^{14}C-TdR for 30 min. 10^{-6} M colchicine was present in the medium throughout the duration of the incubation.

Fig 6 shows the experimental design for the double labelling experiments and the expected results for a normal proliferating cell population. (See WIMBER [20] for discussion of the theory of the double labelling technique in cell kinetics.) The histograms obtained for

the infectious mononucleosis cells are shown in Figs. 9 and 10 The second of these experiments was made with colchicine in the medium to prevent entry of cells into the G_1 compartment. This was done so that the fate of cells that were at the commencement of DNA synthesis (DNA content close to the 2c mode) could be studied

Discussion

The study of the cell kinetics in infectious mononucleosis is complicated by several factors

(a) It is a benign self limiting disease of young people, so apart from the considerations of cost, study of the cells by *in vivo* labelling with ^{3}H TdR is not ethical

(b) The production site of the cells is predominantly in the lymphoid tissues in the lymph nodes there are high mitotic indices (5) The distribution of cells present in the blood in the various phases of the mitotic cycle may be different from that found in the lymphoid tissues. The precise inter relation between the atypical lymphocytes in the blood to those in the fixed lymphoid tissues is uncertain. If there is recirculation from the blood to the lymphoid tissues, then it is possible that sequestration of cells in certain phases of the cell cycle may occur which could be a factor leading to the low mitotic index of the infectious mononucleosis cell in the peripheral blood.

(c) The main part of the population of infectious mononucleosis cells not synthesising DNA is at 2c (G_1) but it is not possible to decide what proportion of it is part of the growth fraction and what proportion is out of cycle in the resting state. In the recovery phase of the disease atypical lymphocytes persist in the blood for several weeks and only a very few of them are synthesising DNA compared to that found in the active phase of the disease (7) Despite these important limitations of experimental design and and subsequent interpretation of results, it is of some value to take stock of what information can be obtained about infectious mononucleosis cells.

It has been previously reported that analysis of the DNA contents of infectious mononucleosis cells that were not labelled with ^{3}H TdR after a 30 minute incubation showed they were all at the 2c mode (G_1) (10) In this present investigation larger samples of cells were examined and a few ... cells were

detected at the 4c mode (G_2) In both of these investigations unlabelled cells were not seen to occur between the 2c and 4c modes in the samples that had been fixed within 1 h after the blood was taken. This suggests that in the blood and during the first hour of culture DNA synthesis continues once it has been initiated since if cells arrested in S they would lie between 2c and 4c and would be unlabelled. This histogram of the distribution of DNA contents of cells in S in the 30 minute labelling experiment (Fig 5) is typical of that found in proliferating cells (4) The very low frequency of cells in G_2 is of interest as it is a strong indication that the rapid transit time of the cells across the G_2 compartment (mean = 1 3 h) as measured *in vitro* has a comparable time *in vivo* since in such a population the number of cells in a given compartment at a given time is proportional to the time spent in that compartment.

The high rate of accumulation of labelled metaphases and the high percentage labelling of the metaphases reached in the cultures without colchicine shows that the cells pass rapidly through G_2 entering M and complete division *in vitro* The similarity of the number of metaphases per 1 000 cells in S after different durations of incubation (Table IV) also suggests that there is no arrest of cells in mitosis.

If we plot the rate of accumulation of labelled metaphases in a sample to which colchicine has been added, then the percentage of labelling of the metaphases will not be the same as that reached without colchicine. This is so because there will be a finite number of unlabelled cells retained at metaphase by the action of colchicine and these will retard the increase in the percentage labelling of metaphases as the radioactive cells accumulate. Fig 3 and 4 show the predicted curves of distribution of colchicine blocked labelled metaphases with increase of time. This curve was determined from the upper one of the rate of accumulation of metaphases without the use of colchicine by the following method

If N_m is the number of unlabelled metaphases after colchicine has been acting for the duration of the G_2 period T is the time of total incubation with colchicine and the radioactive precursor and K_{lm} is the rate of influx of cells into M, then the predicted percentage rate of accumulation of labelled metaphases is

$$\frac{100\,(T\text{-}G_2)\,K_{lm}}{N_m + (T\text{-}G_2)\,K_{lm}}$$

Table III demonstrates that in the cultures there is a rapid expansion of the number of labelled cells in the system when the incubation is continued for 4–5 h. At the same time it is evident that this flux of cells into S is not matched by a comparable flux into M The experiments using the double labelling in conjunction with the analysis of the DNA content of the individual cells has suggested the reason for this rise in the labelling with time. In both cultures there is evidence of a large wave of new cells commencing DNA synthesis during the period from the end of ^{3}H TdR labelling to the time when the culture was fixed. These cells are labelled with ^{14}C alone and form 38% of the labelled cells in Fig 7 and 29% in Fig 8. The cells at the leading edges of these populations had approximately 80% and 75% of the DNA content of the diploid mode. This suggests that in a period of 4½ h they have replicated about 78% of the DNA, which indicates that for the rapidly moving cells the S period is approximately 6 h providing that the initial rate of progress was sustained.

The fate of cells that were in S at the beginning of the culture is illustrated by these two experiments. In the culture in which colchicine was present and cell division did not occur 32% of the cells labelled with ^{3}H TdR during the first 30 min of the incubation were still synthesising DNA 4 h later 29% were tetraploid, having reached G_2 or been arrested in mitosis. The remainder appeared to have arrested their progress through the S period and failed to incorporate the ^{14}C-TdR, despite the fact that the replication of their DNA was incomplete. A similar arrest is seen in Fig 7 Here, as some cell division is taking place, the interpretation of the origin of those cells that are labelled with ^{3}H TdR and whose DNA contents were near to the diploid mode was uncertain. Part of them arise by virtue of the division of labelled mitotic figures and part of the population are cells that have arrested in DNA synthesis.

A failure to divide *in vitro* has been reported to occur in about 50% of the infectious mononucleosis cells that were in S when they were put into cultures (14) The addition of PHA to the medium did not alter the initial division rate of these cells though cultures without PHA ceased to divide after 18–24 h, those with PHA – a small proportion of the infectious mononucleosis cells – continued to divide for 72 h.

The present experiments have given some information about the behaviour of the atypical lymphocytes in infectious mono-

nucleosis. The imbalance between the S and M compartments may be due to effects of cell sequestration – though if this mechanism was operative it would be expected that the mitotic index *in vitro* might rise with time as the cells are unable to leave the culture system.

The dominant feature of the cell kinetics in the culture is the initiation of cells to commence DNA synthesis and the arrest of cells already in synthesis. The induction could be a measure of the true rate of cells moving into the S compartment alternatively it could be the outcome of a stimulation of cells brought about by *in vitro* conditions where they could be freed from homeostatic mechanisms that normally maintain the cells in G_1 Arrest in S may well be due to the *in vitro* conditions – as no evidence of arrested cells were found in those cells that were examined shortly after being removed from the patient. However the alternative explanation must also be considered, that in infectious mononucleosis many cells enter DNA synthesis but only a fraction of them complete the division cycle and divide. If this happens there must be a very rapid removal of effect cells from the circulation. It has been suggested that such a mechanism may be operative in normal myelopoiesis (18)

In summary the main facts that have emerged are as follows (a) Evidence for cell proliferation is confirmed, based on labelling with DNA specific precursors, DNA content of the nuclei and the presence of mitoses (b) There is a steady movement of cells from G_2 into M during the 5 hour culture period (c) There is a marked tendency for cells to commence DNA synthesis shortly after being put into culture. The flux into S is not balanced by a comparable flux into M (d) Cells already at S at the beginning of the culture period may either arrest in S continue to synthesise DNA over a 5 hour period, or complete synthesis and pass through G_2 and M.

These experiments have illustrated some of the intrinsic problems that are to be encountered when the cell kinetics of haematopoietic cells are analysed by *in vitro* techniques. Though some of the potentialities of the dividing cells have been revealed, the relation of these findings to the control of proliferation of infectious mononucleosis lymphocytes that exists *in vivo* remains unknown.

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Summary

The proliferation of infectious mononucleosis lymphocytes *in vitro* has been studied by autoradiographic and quantitative cytochemical techniques. A very small number of these cells were observed to divide during 5 hour period; this group passed through the G_2 period in approximately 1 h without arrest in mitosis. Over a 5 hour period there was marked imbalance between the flux of cells in S and the flux into M, the former being far higher than would have been expected for system in steady state. After 5 h in culture the cells which were synthesizing DNA at the time the blood was taken, had either completed DNA synthesis, arrested in S, or were still synthesizing DNA. A marked induction of the initiation DNA synthesis occurred when the cells were cultured *in vitro* It was considered that the imbalance of the flux rates observed could be accounted for by the combination of the accelerated entry into S and the arrest of cells in S. The relation between the evidence of cell kinetics obtained from the analysis of the cultures to the kinetics *in vivo* is discussed.

Zusammenfassung

Die Proliferation von Lymphozyten der Mononucleosis infectiosa *in vitro* wurde autoradiographisch und quantitativ zytochemisch untersucht. Eine sehr geringe Zahl von Zellen teilte sich innert 5 Stunden; diese Gruppe durchlief die G_2-Periode in ungefähr einer Stunde ohne Arretierung in Mitose. Während eines Zeitraumes von 5 Stunden fand sich eine beträchtliche Abweichung zwischen dem Durchgang von Zellen durch das Stadium S und ihrem Übertritt in die Mitose, indem der erstere größer war als dies für ein System mit gleichmäßigem Ablauf zu erwarten wäre. Nach fünfstündiger Kultur hatten die Zellen, die zur Zeit der Blutentnahme DNA synthetisierten, die DNA-Synthese beendet, waren im Stadium S arretiert oder bildeten weiter hin DNA. Es fand sich eine deutliche Anregung der anfänglichen DNA-Synthese bei der Kultur der Zellen *in vitro* Es wurde angenommen, daß das Mißverhältnis zwischen den Abläufen zu erklären sei durch die Kombination eines beschleunigten Eintrittes in das Stadium S mit einer Arretierung in demselben. Die Beziehung zwischen dem Ergebnis der Zellkinetik in der Kultur und der Kinetik *in vivo* wird diskutiert.

Résumé

La prolifération des lymphocytes dans la mononucléose infectieuse été étudiée *in vitro* à l'aide de méthodes autoradiographiques et cytochimiques quantitatives. Un très petit nombre de ces cellules se divisèrent en l'espace de 5 h; ce groupe passa en à peu près une heure par la période G_2 sans que la mitose ne s'arrêta. Durant ces 5 h, le nombre des cellules passant par le stade S fut beaucoup plus grand que celui passant par le stade M, différence inattendue dans un système au débit constant. Après 5 h de culture, les cellules qui étaient en train de synthétiser de l'ADN au moment de la prise de sang avaient achevé la synthèse de l'ADN ou étaient arrêtées au stade S ou encore continuaient à synthétiser de l ADN Le commencement de la synthèse de l'ADN fut accéléré de façon marquée par la mise en culture *in vitro* des cellules. Les différences

quantitatives du flux des cellules par les différents stades de prolifération pourraient être expliquées par la combinaison d'une entrée accélérée dans le stade S et d'un arrêt des cellules à ce même stade. Les relations existant entre les données sur la cinétique des cellules *in vitro* et *in vivo* sont discutées.

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Depression of the Megakaryocyte Platelet System in Rats by Transfusion of Platelets*

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Information about mechanisms that regulate platelet production is beginning to accumulate. Thrombocytopenia induced experimentally in rats by bleeding or specific antiserum stimulates an increased production of platelets after a lag period of 1 or 2 days, culminating within a few days in a transitory thrombocytosis (1 2) Likewise, transfused serum of platelet-depleted donors can initiate an increase in platelet number (3) During such periods of increased production platelet numbers may exceed normal levels, but they soon return to the normal range. The rapid return to normal suggests the presence of an additional regulatory mechanism that is called into action when platelets exceed a certain level. Indeed, CRONKITE *et al* (4 5) reported that transfusion of large numbers of fresh platelets, sufficient to raise the platelet count of rats to 3 times normal, produced a subsequent depression that appeared 4 or 5 days later when the transfused platelets had disappeared. Platelets remained below normal levels for 3 or 4 days before returning to the pretreatment count. These investigators did not discern any change in the megakaryocyte count in marrow samples taken on the 5th or 7th days after platelet transfusion.

To investigate further this inhibition hypothesis, we have counted the numbers of megakaryocytes in marrow at intervals after platelet transfusions, and have attempted to assess the rate of platelet production by measuring the labelling of platelets with $Na_2S^{35}O_4$

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Methods

Platelet recipients were Sprague-Dawley rats. The body weight of recipients was 231 396 g The weight range in any 1 experiment averaged bout 50 g Donors were larger male rats of the same strain and source.

Blood was collected from the abdominal aorta of anesthetized rats into siliconized syringes containing about 1/10 volume of 1% disodium ethylenediamine tetraacetic acid (Na_2EDTA) in saline. The blood was diluted with saline (5.5) and platelets were separated by differential centrifugation (6) resuspended in saline, and transfused via the jugular vein into recipients within 4–5 h of blood collection. The blood and platelet suspensions were kept in an ice bath or refrigerated centrifuge at 5 C at all times until transfusion. The number of platelets transfused at one time aried from 20×10^{9} to 88×10^{9} usually more than 45×10^{9} in volume that ranged from 2.5 to 6 ml, usually between 3 and 4 ml. About 4.6×10^{9} platelets were obtained from each donor rat; thus 10–19 donors were used per recipient. Transfusion time was about 3 min. In some experiments controls were transfused with an equal volume of saline, but controls in other experiments received no treatment whatever

Platelets of peripheral blood and platelet suspensions were counted by the phase contrast microscopy method (7) Blood for peripheral counts was obtained by pricking the lateral leg vein with 27-gauge needle.

T measure rate of platelet production, platelets were labeled *in vivo* by intra venous (i.) injection of $Na_2S^{35}O_4$, 1 μc/g of body weight (6) Platelets were collected 48 h after isotope injection, washed twice with saline and resuspended in saline. The numbers of platelets in the suspensions were determined and duplicate or triplicate

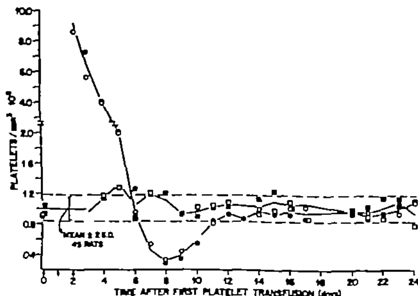


Fig. 1 Peripheral platelet counts of 2 rats transfused on 3 successive days (arrows) with suspension of freshly collected platelets (open and solid circles) or with an equal volume of physiological saline (open and solid squares) Approximately 90×10^{9} platelets in bout 3.5 ml were injected each time. The horizontal lines include 2 standard deviations on each side of the mean platelet count (1.02×10^{6}/mm³) of 45 untreated rats.

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..., as well as plasma and washes, were suspended ... counted in ... liquid-scintillation spectrometer to ... ng medium was made up as follows: 1 liter of 1 4- ... g of PPO scintillator ... nd 0.375 g of dimethyl POPOP ... rmined by using Packard C[14]-toluene standard as an ...

... platelet was computed on the basis of the pretransfusion ... fused rats, since it has been shown that the method labels ... production and therefore transfused platelets would remain ... ount of a suspension was multiplied by the fraction of initial ... (before the experiment) over peripheral count taken just prior to ... resulting value was divided into the radioactivity counts per minute ... suspension to obtain the average radioactivity per platelet. In non- ... used rats, the platelet counts/mm of the suspension were simply divided into the ... activity counts/min/mm[3] of suspension.

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The relative numbers of megakaryocytes were counted in sections of femoral marrow. After excision, the ends of the femurs were cut off for better penetration of the Zenker-formol fixative. The bones were then decalcified for about 2 days, split in half, and embedded in paraffin for sectioning. Ten to 90 high-power fields (average 29) were counted i longitudinal 3–5 μ thick sections stained with hematoxylin and eosin. Marrow sections were scanned moving from left to right in rows through the length of the section, but fields including bone or those only partially filled with marrow were not counted. Megakaryocytes only partially in the field were counted in one half of the field but not in the other. Field diameter was 0.4 mm and magnification was 475 ×.

Results

(1) Effect of platelet transfusion on peripheral platelet count. After a single transfusion of platelets sufficient to raise the circulating level to approximately 3 times normal, the peripheral count steadily declined reaching the normal level between 4 and 5 days later. It then continued below the normal range to its lowest point (75% of normal) at 6 days. At about 9 days it returned to pretreatment level, where it remained for at least the next week.

Platelet transfusions on 3 successive days raised the platelet count to 9 times normal, after which the number of platelets in the peripheral circulation similarly declined to normal in 4 days (Fig. 1) but the subsequent depression was more pronounced than after a single transfusion, declining to about 30% of normal. Again the count remained low for about 4 days and then returned to the normal range. The mean platelet count of 2 rats injected i. v. for 3 days with about 3.5 ml of saline instead of a suspension of platelets rose somewhat above the normal range on the 5th day and then returned to normal within the next few days (Fig. 1).

(2) Effect of platelet transfusion on megakaryocyte number. Megakaryocytes were significantly ($P < 0.01$) reduced in number 3 and

4 days after a single transfusion of 65 to 86 billion platelets, enough to raise the peripheral platelet count 3–4 h after transfusion to 3–5 times normal levels (Table I)

In another series of experiments, platelets were transfused at 0, 24 and 48 hours, and the rats were killed at 72 or 96 h for marrow collection. Transfusion of a total of 143 $\times 10^9$ platelets during a 3-day period, which raised the peripheral platelet count about 3 h after the 3rd transfusion to about 5.5 $\times 10^6$ platelets/mm^3 re *duced the megakaryocyte count at 3 days to about one-half of* that of untreated controls (Table II) The megakaryocyte number was similarly reduced at 3 and 4 days by even larger numbers of platelets.

(3) Effect of platelet transfusion on platelet production ($Na_2S^{35}O_4$ uptake) Platelet labeling during the 48- to 96-hour period after a single transfusion of freshly collected platelets was significantly less ($P < 0.05$) in platelet transfused rats than in controls (Table III) *Furthermore, when* $Na_2S^{35}O_4$ *was injected immediately after the* third of 3 daily transfusions of platelets, the radioactivity of the platelets collected 48 h later differed from that of the controls with a P value of less than 0 01 (Table III)

(4) Circulation of transfused platelets The estimated platelet count within 2–4 h after transfusion of platelets (30 to 90 $\times 10^9$) if all transfused platelets circulated, was calculated by adding to the initial platelet count/mm^3 before transfusion the number of platelets transfused/mm^3 of blood volume. The assumption was

Table I

Megakaryocyte number at intervals after single transfusion of platelets.

Time after platelet injection (days)	Platelets injected $\times 10^9$	Peripheral platelet count $\times 10^6$/mm^3	Meg/HPF[b]	Standard error	No. of rats
–	None	0.96	10.7	0.35	31
1	54–88	3.6–4.9	9 7	0.85	7
3	65–77	3 4–5.0	7.7[c]	0.66	7
4	65–86	4.0–5.1	7.6[c]	0.76	6

This peripheral platelet count was taken 2 ½ to 4 h after transfusion.
Number of megakaryocytes per high-power field. Twelve to 57 fields were counted, usually over 20, in section of femur of each rat.
Using Student' t test, these means differed from that of the control group with P value of < 0.01

Table II

Megakaryocyte number after transfusion of platelets at 0 24 and 48 h.

Group	Total platelets injected $\times 10^{9}$	No. of platelets/injection $\times 10^{9}$	Peripheral platelet counts $\times 10^{4}$/mm^{3}	Meg/HPF[2] Expt.	Meg/HPF[2] Control	Time marrow sampled (day)
I	142.8	43 7 53.8	5.50	4 7	9.9	3
			5.51	3.5	8.1	
II	161.8	48.8–72.2	7 42	6.1	8.8	3
			6.10	5.2	9.2	
			7.30	5.4		
III	196.2	62.0–68.0	7.50	4.9	10.7[3]	4
			7.03	6.5	10.5	
Mean				5.2	9.5	

This peripheral platelet count was taken bout 3-½ h after the 3rd platelet transfusion.
Number of megakaryocytes per high-power field. Ten to 100 fields (usually 30 or more) were counted in section of femur of each rat. Each value is from 1 rat.
The 2 control rats in this group were transfused with 3.2 ml of physiological saline on 3 successive days, whereas the other control rats in this table received no treatment whatever
Control and experimental means differ with P value of < 0.01

Table III

Platelet labelling with $Na_2S^{35}O$ after platelet transfusion.

Platelets injected $\times 10^{9}$	Counts/min/platelet $\times 10^{7}$ Mean	S. E.	No. of rats
none	17 4	0.89	20
86[1]	11.9[3]	1.68	5
240[2]	8.4[4]	1 45	2

[1] Single transfusion of platelets.
Platelets transfused at time 0 24 and 48 hours.
[3] Differed from control with P value < 0.05
Differed from control with a P value < 0.01

made that the blood volume was equal to 6% of body weight. (Values in the literature for blood volumes of rats range from 4 4 to 8.0 ml/100 g of body weight, with perhaps the best values for young adult males being in the 5–6 ml range.) The actual peripheral platelet count 2–4 h after transfusion averaged 86% of the estimated count among 42 rats. This percentage value appeared not to be related to the number of platelets transfused. The percentage of the estimated 2–4 hour platelet count still circulating after 24 h was more variable, having an average of 63%

Discussion

The results of hypertransfusion of platelets on circulating platelet levels confirm the findings of CRONKITE *et al.* (5) that transfusion of large numbers of platelets brings about a subsequent depression of the platelet count. The degree of depression after a single transfusion that raised the circulating count to about 3 times normal was very similar in the two studies. In addition, when the platelet count was raised in our experiments to 9 times normal by 3 daily platelet transfusions, the subsequent depression was much more severe than after a single transfusion that tripled the count, suggesting that the degree of platelet depression is related either to the degree of elevation of the circulating platelets or to the length of time that a high circulating platelet level is maintained. Since the depression was the same in CRONKITE's experiments after a single or 5 daily transfusions that maintained the count at 3 times normal, the magnitude of platelet increase appears to be the important factor.

It is also noteworthy that the platelet count reached normal about 4 days after the last transfusion, the approximate survival time of rat platelets determined by other methods (8). In fact, in the single transfusion experiment the platelet curve crossed the line designating the average normal platelet count at about 4.4 days, in very close agreement with earlier survival time estimates. A slightly earlier arrival of the descending platelet curve at normal level in the more heavily transfused rats (Fig. 1) may reflect a greater or more rapid inhibition of platelet production, thereby falsely accentuating the apparent disappearance rate of the transfused platelets by reducing the number of native circulating platelets.

It is also of interest that there was no suggestion, within the limits of the methods, of oscillation of the circulating levels of platelets after their return to normal following depression, an indication of the presence of effective dampers in this system.

Fewer megakaryocytes in the marrow after transfusion of platelets indicates either (1) a reduction in the number of cells entering the megakaryocyte compartment, or (2) an increase in the number leaving it, or (3) a shorter turnover time in the compartment. One likely explanation is that an abnormally high level of platelets in the circulation may inhibit normal proliferation of

megakaryocyte precursor cells, thereby limiting the entry of new cells into the megakaryocyte compartment. It seems less likely that the exit rate from the megakaryocyte compartment is increased under these circumstances, or that the turnover time is shortened, since such responses would be expected to result from a shortage of platelets rather than from an excess. Additional interpretation awaits a determination of the kinds of cells present in the megakaryocyte compartment in the several days after platelet transfusion. The degree of megakaryocyte depression appears to be quantitatively related either to the number of platelets transfused or to the length of time a high platelet level is maintained, since the megakaryocyte count was more markedly depressed in rats after 3 daily transfusions than after a single transfusion.

Results of earlier *in vivo* platelet labeling experiments have indicated that radioactive platelets in circulating blood 24 or more hours after injection of $Na_2S^{35}O_4$ gain their label during their genesis in the marrow and retain it as long as they survive on the other hand, radioactivity that adheres to circulating platelets immediately upon injection of $Na_2S^{35}O_4$ disappears in less than 24 h (6) The radioactivity of platelets 48 h after injection of $Na_2S^{35}O_4$ should therefore provide an indication of the relative rate of platelet production. The platelet labeling results indicate a reduction of platelet production in rats transfused with large numbers of platelets.

The number of platelets transfused had no apparent effect upon the number capable of circulating 2–4 h later nor was any difference readily apparent due to sampling at different times during the 2–4 hour period after transfusion, although these factors were not systematically explored.

Another point of interest was the disappearance of an average of 23 % of the circulating platelet population in 24 h, a loss that is consistent with previous estimates of platelet life-span of about 4.5 days (8)

The depression of circulating platelet count, megakaryocyte number in the marrow and platelet radioactivity following transfusion of freshly collected platelets indicate that abnormally high numbers of platelets in the circulation inhibit the megakaryocyte-platelet system. The mechanism is not known, but the results suggest a block in the early part of the megakaryocyte cycle, since the rate of platelet disappearance after transfusion does not indicate an immediate cessation of platelet production. In addition, the

megakaryocyte number appears to decline over a period of a couple of days, rather than remaining in an arrested state or very rapidly declining. This is reminiscent of the megakaryocyte response to whole body radiation, where platelet production apparently continues from more mature megakaryocytes but entry of new cells into the megakaryocyte compartment is stopped. One can imagine that platelets produce an inhibitor specific to megakaryocyte precursor cells, the concentration of which regulates division of these precursors and thus entry of cells into the megakaryocyte compartment.

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Summary

Effects of hypertransfusion of platelets on the megakaryocyte-platelet system were investigated, especially in relation to mechanisms regulating platelet production and homeostasis. Rats were transfused with large numbers of platelets, and the number of peripheral platelets, the number of megakaryocytes in the marrow and the rate of platelet production were assessed. The platelet count gradually declined, fell below normal, and then returned to normal. Megakaryocytes were significantly reduced 3 and 4 days after transfusion. Labeling of platelets 48 h after injection of $Na_2S^{35}O$ was also reduced in rats hypertransfused with platelets. The results support the hypothesis that abnormally high numbers of platelets in the circulation inhibit the production of new platelets, possibly by inhibiting proliferation of megakaryocyte precursor

Zusammenfassung

Die Wirkungen der Hypertransfusion von Thrombozyten auf das Megakaryozyten-Plättchensystem wurden untersucht, vor allem mit Bezug auf die Regulationsmechanismen der Plättchenproduktion und der Homöostase. Bei Ratten wurden nach Transfusion großer Zahlen von Thrombozyten die Zahl der peripheren Plättchen und der Megakaryozyten im Knochenmark sowie das Ausmaß der Plättchenproduktion bestimmt. Die Thrombozytenzahl nahm allmählich ab, sank unter die Norm und kehrte dann zur Norm zurück. Die Megakaryozyten waren 3 und 4 Tage nach der Transfusion signifikant vermindert. Die Markierung der Plättchen 48 Stunden nach der Injektion von $Na_2S^{35}O$ war bei den mit Thrombozyten übertransfundierten Ratten ebenfalls vermindert. Die Resultate stützen die Hypothese, daß abnorm hohe Plättchenzahlen im Kreislauf die Bildung neuer Plättchen hemmen, möglicherweise durch Hemmung der Proliferation einer Megakaryozytenvorstufe.

Résumé

Les effets de la transfusion d'un surplus de plaquettes sur le système mégacaryocytes-thrombocytes ont été etudiés particulièrement en ce qui concerne les mécanismes réglant la production de thrombocytes et l'homéostase. De grandes quantités de thrombocytes ont été transfusées à des rats. Le nombre des thrombocytes périphériques et des

mégacaryocytes de la moëlle osseuse ainsi que le taux de production des thrombocytes ont été déterminés. Le nombre des thrombocytes diminua graduellement, tomba en dessous de la normale puis y retourna. Le nombre des mégacaryocytes était nettement diminué le troisième et le quatrième jour après la transfusion. Le marquage des thrombocytes 48 h après l'injection de $Na_2S^{35}O_4$ était également diminué chez les rats ayant reçu un surplus de thrombocytes. Ces résultats étayent l'hypothèse selon laquelle un nombre anormalement élevé de thrombocytes en circulation freine la production de nouveaux thrombocytes, peut-être en inhibant la prolifération d'un précurseur mégacaryocytaire.

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L'index H³ thymidine des mégacaryocytes de la souris intacte et injectée d'hémine

G TVERDY

L'index H³ thymidine des mégacaryocytes médullaires a été établi chez le rat par FEINENDEGEN *et al.* (3) et par EBBE *et al* (2) Ces auteurs ont montré qu une demi heure après l injection intra veineuse de H³ thymidine 20 à 30% des mégacaryocytes du 1er groupe incorporent la substance injectée. Selon la classification de FEINENDEGEN *et al.* le 1er groupe comporte les mégacaryoblastes (18% de l ensemble de mégacaryocytes) le 2d groupe les promégacaryocytes (22 %) et le 3ième groupe des mégacaryocytes mûrs (60%) Ils ont précisé le temps de maturation de mégacaryocytes. Ce temps serait de 40 heures. L observation directe des mégacaryocytes médullaires chez le lapin a permis à KINOSITA (5) d'établir la durée de vie d un mégacaryocyte à 5 jours. CRONKITE *et al.* (1) ont étudié l'incorporation de H³-thymidine chez la souris.

Nous nous sommes demandé si l index H³ thymidine des mégacaryocytes médullaires et spléniques de la souris était identique et s il ne variait pas sous l effet d un stimulant approprié. Les facteurs qui stimulent la formation de mégacaryocytes, plus particulièrement de mégacaryocytes extra médullaires, et augmentent le nombre de ces cellules sont multiples. Citons la nécrose cellulaire et l'hémolyse. Plusieurs porphyrines, entres autres l'hémine, ont un même effet. Lorsque le nombre de mégacaryocytes extra médullaires s accroît, l index H³ thymidine pourrait subir une modification Nous l avons étudié d abord chez la souris intacte et ensuite chez la souris injectée d'hémine Au cours de cet exposé nous montrerons d abord l'effet d'hémine sur l accroissement du nombre de mégacaryocytes extra médullaires et nous

établirons ensuite l index H³ thymidine des mégacaryocytes spléniques et médullaires chez la souris intacte et traitée à l hémine.

Matériel et méthode

1 Nous vons utilisé dans cette première partie de notre travail, 50 souris mâles de souche pure âgées de 4 à 9 mois. Quarante souris ont servi de témoins. Dix autres ont été injectées d'hémine par oie sous-cutanée. Chaque injection comportait 2,5 mg d'hémine qui était dissout dans NaOH à 1‰. Trois injections ont été faites, chacune à deux jours d intervalle et les animaux ont été sacrifiés sept jours après le début de l ex périence. Sur le foie de ces 50 souris nous vons tracé à l'encre de Chine des carrés de 0,25 cm de surface et compté le nombre de mégacaryocytes dans chacun de ces carrés.

Pour comparer le nombre de mégacaryocytes hépatiques chez les animaux témoins et les nimaux d expérience et établir un accroissement statistiquement valable on peut se servir de la formule ci-dessous, lorsque la distribution cellulaire est celle de Poisson.

$$P = 1-\left(\frac{N}{N+N_1}\right)^{(\ +1)}\left[1+\frac{(\ +1)N}{1!(N+N_1)}+\frac{(\ +1)(\ +2)}{2!}\left(\frac{N_1}{N+N}\right)^2+\right.$$
$$\left.\ldots+\frac{(\ +1)(\ +2)\ldots(\ +\ )}{!}\left(\frac{N}{N+N_1}\right)\right]$$

ou N est le nombre d carrés examinés chez les animaux témoins,
ou N est le nombre de carrés examinés chez les animaux d expérience
ou est le nombre de mégacaryocytes trouvés dans N carrés,
ou est le nombr de mégacaryocytes trouvés dans N carrés.

Dans la rate une pareille ppréciation n est pas possible. L pparition de mégacaryocytes dans les follicules lymphoïdes, toujours dépourvus de ces cellules à l'état normal, peut servir de critère pour déterminer un accroissement de mégacaryocytes spléniques.

2 L index H³-thymidine été établi chez trois souris mâles du même élevage du même âge et d même sexe que ceux qui ont servi à l'expérience précédente Deux souris intactes ont serv pour établir l'index H³-thymidine des mégacaryocytes médullaires et spléniques et une souris pour déterminer le même index dans la rate près l'injection d'hémine

L injection d'hémine été fait selon le schéma indiqué plus haut. L'H³-thymidine été injectée par voie intra-péri onéale à raison de 1,5 µC par g de poids et les animaux ont été sacrifiés 60 min plus tard. L injection se faisait toujours même moment de la journée Les organes ont été fixés dans un mélange de formol (40 parties) d alcool (40 parties) et d'acide acétique glacial (20 parties) Les préparations histologiques ont été colorées selon la méthode de Feulgen, recouvertes d'un émulsion Ilford K_5 et gardées à la glacière pendant 28 jours à une t de 20

Resultats

1 L examen de quarante foies des souris témoins a permis de trouver en tout 4 mégacaryocytes 36 carrés étaient dépourvus de ces cellules et quatre autres comportaient une cellule par carré. La moyenne était de 0 1 mégacaryocyte par carré. Dans dix carrés des souris injectées d hémine nous avons trouvé en tout huit méga caryocytes. Quatre carrés ne comportaient aucun mégacaryocyte,

quatre carrés étaient munis d un mégacaryocyte et deux carrés montraient deux cellules. La moyenne s est ainsi établie à 0 8 mégacaryocyte par carré. La formule ci-dessus nous permet de constater que *l accroissement de la moyenne est statistiquement* valable. L injection du solvant seul n a pas accru le nombre de mégacaryocytes.

2 La lecture de nos autoradiographies nous a permis d établir des données du tableau I

L examen histologique permet de constater que le nombre de mégacaryocytes hépatiques et spléniques augmente après l in jection d hémine. En effet, non seulement les mégacaryocytes spléniques sous-capsulaires deviennent plus nombreux, mais ils apparaissent dans les follicules lymphoïdes de la rate. Les cellules réticulaires spléniques marquées par la thymidine sont plus nombreuses après l injection d'hémine, alors que l'index mitotique *de ces cellules reste bas. Les mitoses qui apparaissent dans les* noyaux mégacaryocytaires après l'injection d hémine et de thymi dine ne sont pas marquées.

Discussion

L accroissement du nombre de cellules réticulaires spléniques marquées, sans accroissement d'index mitotique indique une orientation probable de ces cellules vers la polyploïdie.

L index H^3 thymidine des mégacaryocytes médullaires est in férieur à celui des mégacaryocytes spléniques. Il est probable que le cycle générateur des mégacaryocytes médullaires et spléniques ne se déroule pas d une manière tout à fait superposable Par

Tableau I

	Injection de H^3-thymidine		
	Souris témoins		Souris injectées d'hémine
	Mégacaryocytes		
	) médullaires	b) spléniques	) spléniques
Nombre de noyaux examinés	1000	700	1000
Pourcentage moyen des noyaux marqués	4 8 (3 6)	11,5 (7 17)	11,5 (8 18*)

contre, cet index ne change pas après l'injection d'hémine. En d'autres mots, si l'hémine accroît le nombre de mégacaryocytes hépatiques et spléniques, cette substance ne modifie pas pour d'autant le temps de leur maturation. Nos pourcentages ont été établis sur l'ensemble de mégacaryocytes. Comme seuls les méga-caryoblastes incorporent la thymidine, il faut introduire une correction pour comparer nos résultats à ceux de Feinendegen (3) et Ebbe (2). Nous voyons alors que le pourcentage de mégacaryocytes médullaires marqués est approximativement le même chez le rat et la souris 30 à 60 min après l'injection de la thymidine.

Comme la littérature nous le montre, plusieurs questions restent encore irrésolues. Selon Cronkite (1) il arrive que seuls certains lobes des noyaux mégacaryocytaires incorporent l'H^3-thymidine, alors que d'autres restent non marqués. Dans ces conditions, l'index H^3 thymidine ne peut pas être établi d'une manière aussi uniforme que celui des noyaux diploïdes. Ebbe observe cependant rarement de telles images. Nous pensons qu'elles doivent être assez exceptionnelles. Il s'agit peut être d'une superposition de noyaux marqués réticulaires ou autres au-dessus d'un lobe du noyau mégacaryocytaire.

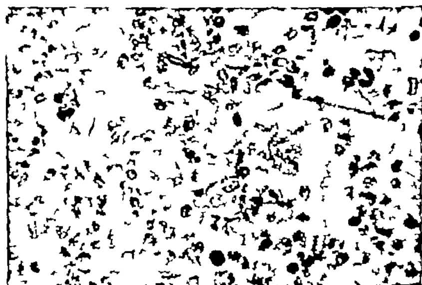


Figure. Souris injectée d'hémine. Rate. D'un côté de la préparation, présence d'un mégacaryocyte marqué par la H^3-thymidine, de l'autre côté un mégacaryocyte non marqué. Nombreuses cellules réticulaires marquées par la thymidine. Coloration à l'hémalaun-éosine.

Les recherches récentes ont montré que la charge finale en DNA varie d'un animal à l'autre. C'est ainsi que chez le cobaye la charge nucléaire finale serait de 32n (5) alors que chez le lapin elle serait de 64n(4). Dans ces conditions, le temps de maturation pourrait être variable d'une espèce animale à l'autre. Les autoradiographies montrent que pratiquement les mégacaryoblastes seuls incorporent la thymidine. Dans ces conditions, la charge nucléaire finale en DNA (32 et 64 n) devrait apparaître dans les mégakaryoblastes avant qu'ils ne deviennent des promégacaryocytes et les mégacaryocytes. C'est l'hypothèse défendue actuellement par ODELL *et al.* (6).

Le temps disponible à la fixation de H^3 thymidine par le noyau qui procède à sa synthèse en DNA est réduit à 40 min (MAURER). Or FEINENDEGEN *et al.* ainsi que EBBE *et al.* constatent que plusieurs heures et jours après l'injection de la thymidine le nombre de noyaux mégacaryocytaires marqués ne cesse de croître. Selon FEINENDEGEN *et al.* les cellules souches des mégacaryoblastes synthétiseraient le DNA pendant un temps particulièrement long (1 à 3 jours). EBBE *et al.* pensent, par contre, que la thymidine fixée au préalable par les érythroblastes pourrait être mise plus tardivement à la disposition de l'organisme et serait incorporée par les cellules non marquées au début de l'expérience.

Résumé

L'hémine accroît le nombre de mégacaryocytes extra-médullaires. Il est vraisemblable qu'il stimule la transformation de cellules souches en mégacaryoblastes. La formation accrue de mégacaryocytes ne va pas de pair avec une modification de l'index H^3-thymidine. Par contre, cet index n'est pas le même pour les mégacaryocytes médullaires et extra-médullaires chez une souris intacte.

Il est probable que le cycle générateur des mégacaryocytes ne se déroule pas d'une manière identique au niveau de la moelle et de la rate.

Summary

It is shown that haemin increases the number of extra-medullary megakaryocytes. It most probably stimulates the transformation of stemcells into megakaryocytes. The increased formation of megakaryocytes does not parallel the modification of the H^3-thymidine index. On the other hand, in intact mouse, this index is different in medullary and extra-medullary megakaryocytes.

It is suggested that the generatory cycle of megakaryocytes in the bone-marrow and in the spleen does not follow exactly the same pattern.

Zusammenfassung

Hämin bewirkt eine Zunahme der extramedullären Megakaryocyten. Wahrscheinlich stimuliert es die Umwandlung von Stammzellen in Megakaryoblasten. Die gesteigerte Bildung von Megakaryocyten geht nicht mit einer Änderung des H³ Thymidin-Index einher. Dagegen ist dieser Index für medulläre und extramedulläre Megakaryocyten der normalen Maus nicht gleich. Es ist möglich, daß der Zyklus der Megakaryocytenbildung in Knochenmark und Milz nicht in gleicher Weise abläuft.

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Studies on Erythropoietic Action of Testosterone

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The erythropoiesis stimulating effect of androgen has already been established by the observation of GORDON *et al.* (1) that long term administration of androgen resulted in a significant increase in erythrocyte count of either castrated or hypophysectomized rats. Recently clinical effectiveness of this hormone in the treatment of hypoplastic anemia was demonstrated (2–4). These facts have promoted research work on the relation of this hormone to erythropoiesis. However, the exact mechanism of its stimulating effect on the erythropoiesis has been unsettled.

In this paper, the results of our own experiments attempted to investigate the nature of erythropoietic action of androgen are described.

Materials and Methods

Albino rats of Wistar strain weighing 130–150 g were used in these experiments. Group A consisted of 34 castrated male rats, group B of 24 normal intact male rats, group C of 35 normal intact female rats, group D of 30 normal intact female rats, group E of 43 intact normal female rats and group F of 67 polycythemic female rats. Polycythemia (Ht 70%) was obtained in these rats by intraperitoneal injection of homologous erythrocytes suspended in normal saline. Castration was carried out in the animals of group A 5 days prior to the initiation of experiment.

Testosterone-enanthate in sesame oil in concentration of 100 mg per ml (enarmon-Depo®) was used as androgenic hormone. Testosterone treatment was carried out as follows: one tenth ml of Enarmon-Depo® was injected intramuscularly in experimental rats of either group A, D, E or F once every 7 days for 60 days. As control, rats of group B, C and part of rats of group A were treated with equal amount of sesame oil instead of Enarmon-Depo® injection.

Quantitative determination of erythropoiesis was performed as follows: rats were injected with 0.3 μc of radioiron ($^{59}FeCl_3$, specific activity 17.0 mc/mg) in 0.3 ml of normal saline intravenously. Eighteen hours later 1.0 ml of blood was obtained by means of cardiac puncture and the radioactivity of erythrocyte mass was determined with the well-type scintillation counter. Radioactivity in total red cell mass was expressed

in percentage to the amount of ^{59}Fe injected, assuming the total blood volume of experimental rats is 5% of the body weight.

Plasma samples for assay of erythropoietin activity were obtained as follows five to fourteen animals of group E or F were sacrificed by exsanguination at various days after the initiation of the testosterone treatment and plasma obtained from rats of these groups were pooled. All pooled samples were stored at —20°C until bioassay of the erythropoietin activity Activity of erythropoietin in the pooled plasma was bioassayed as follows: female rats of Wistar strain, weighing 140 150 g, were used as bioassay animals. Two ml of the pooled test plasma was injected subcutaneously to each of four to six fasted rats once a day for two days. Three tenth μc of $^{59}FeCl_3$ was injected intravenously 24 h after the second injection of the test plasma. Percentage of ^{59}Fe utilized by red cell mass in 18 h was calculated similarly as above described.

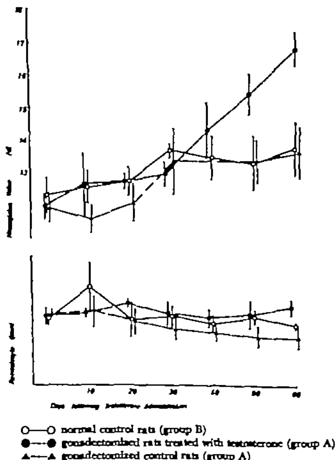
Basal metabolic rate was determined by using the method described by OGATA and TANAKA (5) The amount of CO_2 expired from the rat placed in closed chamber was measured as a parameter indicating basal metabolic rate. Carbon dioxide expired from the rat was completely adsorbed by passing through a bottle containing a given amount of NaOH solution. Then the CO_2 content in this solution was determined by titration with HCl. Basal metabolic rate was expressed as mg CO_2 expired per hour per g of body weight.

Hemoglobin concentration and hematocrit were measured by cyanmethemoglobin method and capillary tube method, respectively Circulating blood volume and apparent survival time of peripheral erythrocytes were determined by ^{51}Cr-tagging method. Concentration of iron and copper in serum was determined with the method of LANDERS and ZAK (6) and that of free proto- and copro-porphyrin with the method of SCHWARTZ and WIKOFF (7)

Results

(1) Erythropoiesis stimulating effect of testosterone administration for 60 days Changes in both hemoglobin levels and reticulocyte counts in 17 castrated male rats of group A during 60 days of testosterone treatment are shown in Fig 1 The other 17 castrated male rats of group A and 24 normal intact male rats of group B served as control. In the rats treated with testosterone, a significant increase in hemoglobin concentration was observed while no changes in control rats of either group A or B There were no changes in reticulocyte counts as percentage, however increase in reticulocyte count per mm^3 was evident in testosterone-treated rats, since erythrocyte counts increased gradually in these rats.

Results of blood counting ferrokinetic study survival time of erythrocytes, serum iron and copper levels, content of free proto- and copro-porphyrin in erythrocytes determined at the end of 60 day period of experiment are listed in Table I A significant increase in circulating erythrocyte mass and serum copper level was observed in castrated male rats treated with testosterone. In these animals the serum iron level decreased and more radioiron was utilized by erythrocytes. There was no significant difference in these



O——O normal control rats (group B)
●——● gonadectomized rats treated with testosterone (group A)
▲——▲ gonadectomized control rats (group A)

Fig. 1 Changes of hemoglobin levels and reticulocyte counts in castrated male rats during 60 days of testosterone treatment and in normal or castrated control male rats during the same period of treatment with sesame oil.

values between the control rats of group A and B. From these results, it can be concluded that testosterone administration stimulates erythropoiesis in castrated male rats.

In these experimental animals, a marked increase in weight of the adrenal gland was noted in the control rats of group A and testosterone treatment to thus castrated rats of group A prevented an increase of the weight of the adrenal gland.

(2) Relation of increase in ^{59}Fe-utilization of red cells to the basal metabolic rate in the rats during 60 days of testosterone treatment. Basal metabolic rate was estimated on 5th, 10th, 15th, 18th and 21st day

Table I

Various hematological data obtained at the end of 60 days of experimental period.

Treatment	Sesame oil		Testosterone enanthate in sesame oil
	Normal male control rats (group B)	Castrated male control rats (group A)	Castrated male testosterone-treated rats (group A
ght (g)	256.0 ± 8.7 (8)	228.0 ±15.6 (8)	211.0 ±11.5 [illegible]
bin (g/dl)	13.6 ± 0.8 (8)	13.5 ± 0.8 (8)	16.7 ± 0.. [illegible]
yte count (%)	3.7 ± 0.2 (8)	3.0 ± 0.7 (8)	4.8 ± 0.5 [illegible]
%)	50.0 ± 6.0 (8)	28.6 ± 0.7 (8)	50.0 ± 0.5 [illegible]
g total RBC volume ; BW)	2.53 ± 0.18 (4)	2.49 ± 0.06 (4)	3.14 ± 0.0 [illegible]
ell utilization (%)	1.5 ± 5.8 (8)	70.0 ± 3.6 (5)	31.5 ± 2.7 [illegible]
time of RBC (days) ging method T ½)	10.3 ± 0.. (8)	11.3 ± 0.2 (4)	10.6 ± 0.5 [illegible]
n (µg/dl)	1 .0 ±38.2 (6)	.14.5 ±15.2 (5)	140.. ±24.5
pper µg/dl)	151.3 ± 4.9 (6)	146.7 ±18.3 (5)	235.0 ±27.8
hrocyte protoporphyrin (µg/dl RBC)	32.9 ±10.7 (4	4..8 ±10.8 (4)	48.5 ±10.6
hrocyte coproporphyrin BC)	3.59 ± 1.23 (4)	4.0 ± 1..4 (4)	4.59 ± 1.9 [illegible]
f adrenal glands (mg/100 g BW)	18.3 ± ..0 (5)	26.5 ± 2.0 (5)***	15.7 ± 0.9 [illegible]

(Mean ± S. E. of the m

in parentheses represent the number of rats in each group.

ically significant difference (P <0.01) between testosterone-treated and castrated control male rats p A.

ically significant difference (P <0.05) between testosterone-treated and castrated control male rats p A.

ically significant difference (P <0.05) between normal male rats of group B and castrated control of group A.

after the initiation of testosterone treatment in thirty female rats of group D. Changes in erythropoietic activity were investigated in these rats of both group C and D. Utilization of ^{59}Fe by red cells was determined on 9th, 15th, 21st, 30th, 40th and 60th day following the initiation of the experiment. Five or six rats were subjected on each determination of ^{59}Fe-utilization. Utilization study was carried out only once in each of either testosterone-treated or control rats during 60 day period of observation. As shown in Fig. 2, an evident increase in ^{59}Fe-utilization as a response to testosterone administration was observed on 15th and 21st day and thereafter the percentage of ^{59}Fe utilized by red cells decreased gradually even in testosterone treated rats. However, higher levels remained as compared with that of control rats throughout 60 days of the

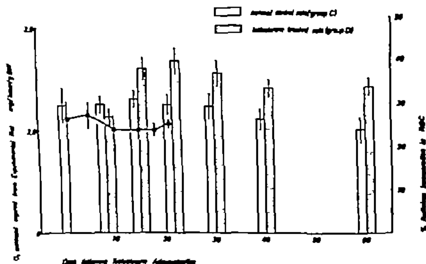


Fig 2. Changes of [59]Fe-utilization by red cells and basal metabolic rate in normal female rats during 60 days of testosterone treatment. Each bar represents an average [59]Fe-incorporation to red cells in percentage with a range of the standard error The interrupted line shows an average fluctuation of BMR in the rats of group D with range of the standard error

experimental period. No remarkable change was observed in the basal metabolic rate during the initial 21 days of the experiment, in contrast to a marked elevation of erythropoiesis.

(3) Activity of erythropoietin in plasma obtained from testosterone-treated rats The activity of erythropoietin in pooled plasma obtained from normal rats of group E or polycythemic rats of group F at various times following the initiation of testosterone treatment was bioassayed. The results are shown in Table II. There was no increase in erythropoietin activity in plasma obtained at any time of 30 days of the experimental period from the animals of either experimental group

Discussion

It is evident from the results that testosterone stimulates erythropoiesis not only in castrated male rats but also in normal female rats. This is in contradiction to the observation of FRIED *et al.* (8) that testosterone produced no detectable changes in hemoglobin production and [59]Fe utilization by red cells in normal mice. It has already been demonstrated that the erythropoiesis stimulating

Table II
Erythropoietin level of plasma obtained from normal rats of group E and polycythemic rats of group F treated with testosterone for various days.

Duration of testosterone administration days	Plasma erythropoietin level of testosterone-treated rats (group E) %	Plasma erythropoietin level of testosterone-treated polycythemic rats (group F) %
0	6.4±0.7 (5)	4.0±0.9 (5)
5	5.4±1.0 (5)	6.4±0.8 (5)
10	4.2±0.8 (6)	5.0±0.8 (6)
15	8.2±1.1 (5)	6.5±0.4 (5)
20	7.2±1.4 (6)	4.5±0.9 (5)
30	6.9±1.2 (4)	3.5±1.8 (4)

(Mean ± S. E. of the mean)

Numbers in parentheses represent the number of bioassay animals.

effect of testosterone is more striking in castrated or hypophysectomized animals (1) and is slight in normal animals (9). However it should be considered from the results obtained in the present experiment that castration has been functionally compensated in certain degree by the hypertrophy of the adrenal gland.

The decrease of serum iron observed in testosterone-treated animals can be interpreted as a result of an increase in erythropoietic activity. It is not clear whether hypercupremia in testosterone-treated rats was related to an enhancement of erythropoiesis. Further studies are required to answer this question. It is of importance that no shortening of survival time of erythrocytes was observed in the rats treated with testosterone, taking into consideration STOHLMAN's observation (10) that red blood cells formed in response to erythropoietin have a substantially short life span.

Stimulation of the erythropoietic activity by testosterone administration was not accompanied by an increase in oxygen consumption. This fact indicates that testosterone does not exert a stimulatory effect on erythropoiesis indirectly through an elevation of the basal metabolic rate. It is suggested that the increase in erythropoiesis by testosterone treatment might result from an elevation in erythropoietin activity, although we failed to confirm an increase of erythropoietin activity in plasma from either normal or polycythemic rats treated with testosterone. GORDON *et al.* (11) have recently reported that the injection of testosterone increases the erythropoietin activity of plasma of mice and rats. Thus, the question

arises whether the procedure using for the bioassay of erythropoietin activity in our experiment was sensitive enough to detect an increase in erythropoietin activity in plasma samples. GURNEY *et al.* (12) have indicated that the hypertransfused polycythemic mice are the most sensitive bioassay animals for the determination of erythropoietin activity especially for small amounts of erythropoietin. It might be possible to prove an increase in activity of erythropoietin in plasma from testosterone-treated rats, if the hypertransfused polycythemic mice were used as bioassay animal. However it should be considered that testosterone might potentiate the effectiveness of small amounts of endogenous erythropoietin elaborated in testosterone-treated rats or that its stimulatory effect on erythropoiesis might be based on a erythropoietin-like action of testosterone.

Summary

A long term administration of testosterone accelerates ^{59}Fe-utilization by red blood cells and increases the circulating red blood cell volume and hemoglobin in castrated male rats. Acceleration of ^{59}Fe-utilization by testosterone is observed in normal female rats without elevation of the basal metabolic rate. W failed to demonstrate an increase in erythropoietin activity in the plasma of normal or hypertransfused polycythemic rats treated with testosterone.

Zusammenfassung

Bei kastrierten männlichen Ratten führt eine langdauernde Zufuhr von Testosteron zu einer Beschleunigung der Eisenverwertung durch rote Blutkörperchen und zu einem Anstieg von zirkulierendem Erythrozytenvolumen und Hämoglobin. Eine Beschleunigung der Eisenverwertung durch Testosteron findet sich bei normalen weiblichen Ratten ohne Steigerung des Grundumsatzes. Im Plasma normaler oder übertransfundierter polyzythämischer Ratten konnte nach Testosteronbehandlung keine Zunahme der Erythropoietinaktivität nachgewiesen werden.

Résumé

L administration à long terme de testostérone accéléra l'utilisation de ^{59}F par les érythrocytes et augmenta le volume érythrocytaire circulant ainsi que le taux d'hémoglobine chez des rats mâles châtrés. L accélération de l'utilisation de ^{59}F par la testostérone n était pas accompagnée chez des rates normales d'une augmentation du métabolisme de base. Une augmentation de l'activité de l'érythropoïétine ne put pas être démontrée dans le plasma de rats normaux ou de rats rendus polycythémiques par des transfusions de sang près un traitement à la testostérone.

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Paroxysmal Nocturnal Haemoglobinuria Following 'Aplastic Anaemia

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Paroxysmal nocturnal haemoglobinuria (PNH) is an uncommon, acquired disorder of undetermined aetiology characterised by chronic haemolytic anaemia, constant haemosiderinuria and occasionally by bouts of haemoglobinuria, which are classically nocturnal. These manifestations are due to the appearance of a proportion of intrinsically defective cells, the presence of which in the peripheral red cell population can be demonstrated by undue sensitivity to lysis *in vitro* by acidified serum (Ham's test) or after the further addition of thrombin (Crosby's test)

Leucopenia and thrombocytopenia of moderate degree are frequent findings, usually associated with a morphologically normal or hyperplastic bone marrow Some patients with PNH have severe peripheral pancytopenia associated with bone marrow hypoplasia and this may occur during the course of the disorder (1 9) In other patients the period of aplasia may precede the diagnosis of PNH (2, 3 4 7 10 11) Dacie (2) has suggested that somatic mutation may occur during the regenerative phase following a period of bone marrow suppression, producing a clone of PNH cells.

The purpose of this short communication is to put on record another example of this sequence of events. The patient was a girl of 19 years whose 'Idiopathic aplastic anaemia was supported for 21 months by blood transfusions and other treatment. Her apparent recovery was found to be associated with the appearance of the PNH erythrocytic defect which has persisted to date.

Case Report

A 19 year old female student, who had neither taken drugs nor been exposed to chemicals nor radiation, was in normal health until 1st February 1964, when she complained of a sore throat. She was confined to bed and given a one week course of penicillin in standard dosage with symptomatic improvement. As she appeared anaemic course of oral iron was administered. After two weeks there was no clinical response and the presence of bruises was noted on the limbs.

On 1st February 1964 three weeks after she complained of sore throat, she was admitted to the Royal Victoria Hospital, Belfast, for investigation. On examination the patient appeared anaemic but not icteric. There were no exudative or ulcerative lesions in the mouth and the tonsils ppeared normal. Bruises, probably traumatic, were present over the lower limbs but no purpuric spots were seen. There was no lymphadenopathy splenomegaly nor other bnormality Laboratory studies revealed haemoglobulin of 4.8 g/100 ml, reticulocytes 2% platelets 46,000 mm^3 leucocytes 2,800 mm^3 with 16 neutrophils and 84 lymphocytes. Stained peripheral blood smears showed moderate erythrocyte anisocytosis and macrocytosis. A sternal marrow aspirate was extremely hypocellular with gross reduction of all three prime elements. On the basis of this evidence diagnosis of idiopathic aplastic anaemia was made and the patient was given transfusion of whole blood following which prednisone therapy was instituted.

After fourteen day course of prednisone in dosage of 40 mg daily had produced neither clinical nor haematological response the dose was reduced to 10 mg daily As bleeding from the gums, epistaxis and excessive menstrual loss persisted the prednisone was discontinued after 6 weeks. Following severe menstrual loss in May 1964 three units of platelet-rich whole blood were prepared for transfusion. When about 100 ml, of the first unit had been given an urticarial rash appeared. This responded to adrenalin and the transfusion was continued. Two further units of platelet-rich whole blood were transfused under anti-histamine cover without reaction. Routine post-transfusion investigation revealed the presence of leucoagglutinins in the serum. Subsequent whole blood transfusions produced no reaction when hydrocortisone was administered over the transfusion period.

Because of the severe menstrual blood loss which was difficult to control, it was decided to induce temporary amenorrhoea and accordingly in June, 1964 the patient was started on norethisterone ("Primolut N") in standard dosage. After a menstrual period in July 1964 which lasted two weeks, menstruation ceased. In spite of the elimination of this source of blood loss the patient still required transfusion of two to four units of blood t intervals of three to four weeks. Combined haematinic therapy with cyanocobalamin, pyridoxine, folic acid and further androgen therapy in the form of testosterone ("Sustanon 250 mg every two weeks, produced no clinical or haematological improvement.

In March, 1965, the patien again complained of sore throat with hoarseness, and was found to have small ulcer in the right upper edge of the epiglottis. A chest -ray showed no abnormality No acid and alcool fast bacilli were seen on direct examination of gastric washings, sputum and urine. Cultures for Mycobacterium tuberculosis were negative The Mantoux test was negative 1 10 000, positive 1 1,000. Although there was no evidence of tuberculosis, the possibility remained that the ulceration of the epiglottis and the plastic anaemia were due to non-reactive tuberculosis. For this reason therapeutic trial of calcium benzamidosalicylate and isoniazid ("Theraxid") in standard dosage was instituted and treatment with "Sustanon and haematinics discontinued. One month after the commencement of "Theraxid" therapy the ulcer on the epiglottis was found to be healed.

A period of 9 weeks elapsed before the patient again required transfusion. This was in June, 1965 when the reticulocyte count was found to be 12% although leucopenia and thrombocytopenia were still present. The reticulocytosis, together with the fact that the patient had previously required *transfusion every three to four weeks, suggested* that haemopoietic regeneration was taking place. A bone marrow aspirate was obtained but again found to be hypoplastic. Four units of platelet-rich blood were transfused. This proved to be the last transfusion that the patient required as the haemoglobin was subsequently maintained in the region of 8—10 g/ 100 ml, although the leucocytes remained less than 3,500 mm³ and the platelets less than 60,000/mm³

The reticulocytes, apart from transient fall following the last transfusion, remained greater than 10% and this observation prompted further laboratory studies. In December 1965, the haemoglobin was *10.2 g/100 ml, PCV 31% MCHC 33* reticulocytes 9.5% platelets 70 000/mm³ leucocytes 3500/mm³ with neutrophils 21% lymphocytes 73% and monocytes 6%. Stained peripheral blood films showed erythrocyte anisocytosis and slight macrocytosis and on staining by the method of Hayhoe and Quaglino (5) reduced leucocyte alkaline phosphatase was noted. Red cell fragility of fresh blood normal. Heat resistance on clotted blood showed gross haemolysis after one hour at 37 °C. Red cell acetylcholinesterase was at the lower limit of normal, the cholinesterase number being 30 by the method of Michel (7) normal range 51—100. No haemoglobin-haptoglobin complex was detected electrophoretically in serum to which carboxyhaemoglobin solution had been added. Serum bilirubin 1.2 mg % acidified-serum (Ham's) test positive, hot-cold lysis (Donath-Landsteiner) test negative, direct Coombs test and serological tests for syphilis negative. On examination of the urine no protein, bile, sugar or blood was detected but urobilinogen was present in excess. Microscopical examination of the urinary deposit revealed the presence of haemosiderin which gave positive Perl's prussian blue reaction.

The presence of haemolysis was indicated by the reticulocytosis, absent serum haptoglobins, increased serum bilirubin, excess urinary urobilinogen and significant haemosiderinuria. A positive heat resistance test pointed to possible diagnosis of PNH and this was confirmed by Ham's test.

Since PNH was diagnosed, the patient has been reviewed monthly and has remained clinically well. Gross haemoglobinuria has never occurred, and chemical tests for blood in the urine have been persistently negative. The haemoglobin has remained in the range of 7—10 g/100 ml, reticulocytes 2.5—10%, platelets 60000—100000/mm³ and leucocytes 3,000—4,000/mm³ of which less than 1,200/mm³ have been neutrophils. On each review haemosiderinuria has been present and the heat resistance test on clotted blood has been positive.

Comment

This case presented as apparent idiopathic aplastic anaemia and the more usual causes of bone marrow suppression were excluded. A possible diagnosis of pancytopenia associated with nonreactive tuberculosis was seriously considered both at the onset and in a later phase when an epiglottal ulcer had developed. However there was neither clinical nor laboratory evidence to support this diagnosis. Despite this a course of anti-tuberculous therapy was given on empirical grounds and on this treatment apparent bone marrow recovery occurred.

For 21 months the patient was maintained on a blood transfusion programme during the course of which she received transfusion of three to four units, mainly of whole blood, at approximately three to four weekly intervals, to a total of 57 units. In order to prevent menstrual blood loss, menstruation was temporarily suppressed by the administration of norethisterone. Various courses of haematinics, including cyanocobalamin, folic acid, pyridoxine and androgens were given without benefit and the pancytopenia persisted without evidence of any excessive haemolysis. After the patient had been on this supportive regime for 17 months the presence of 12% reticulocytes was found in the peripheral blood. The possibility that this represented active bone marrow regeneration was considered but the bone marrow aspirate still showed hypoplasia and the reticulocytosis disappeared following the depressant effect of a further transfusion of blood. Recovery from the aplasia appeared to begin at this time as this was the last blood transfusion that the patient required. Four months later the reticulocytosis reappeared and persisted. Laboratory studies then showed evidence of intravascular haemolysis associated with the characteristic erythrocytic defect of PNH The urine also showed constant haemosiderinuria, although haemoglobinuria was never detected.

The evidence of recovery from aplastic anaemia in this patient was somewhat marred by the demonstration of the PNH erythrocytic defect. In the case conforms to the normal pattern of the life history of PNH many problems will arise during management (6) which cannot fail to make the outlook gloomy However in some patients the degree of haemolysis may not be severe and on the very rare occasion complete clinical and haematological recovery has been described (2)

In view of the number of reported cases, there appears to be a relationship between bone-marrow aplasia and PNH, which is not due to pure chance. Phases of bone-marrow hypofunction during the course of PNH (1 9) could be explained on the basis of the functional failure of an over active marrow particularly in the event of deficiency of essential metabolites.

In the majority of cases presenting as aplastic anaemia (2 3 4 7 10 11) there was no clinical or haematological evidence of PNH during the aplastic phase. The case which we have described appears to be another exmple of this sequence of events. Although

specific tests designed to detect the erythrocytic defect of PNH were not performed there was neither evidence of intravascular haemolysis, nor was haemolysis induced by the transfusion of plasma or whole blood during the period of bone marrow aplasia. The current view of the pathogenesis in this type of case has been put forward by DACIE (2) who considers that PNH may occur as a complication of the aplastic anaemia, a somatic mutation during the regenerative phase leading to the development of a clone of PNH cells in the marrow

Acknowledgement. We wish to thank Dr J T Lewis for some clinical information about this patient.

Summary

The case of a 19 year old girl is described, in whom apparent recovery from idiopathic aplastic anaemia after 21 months was followed by the appearance of the PNH erythrocytic defect.

Zusammenfassung

Es wird über ein 19 Jahre altes Mädchen berichtet, bei dem im Anschluss an die Remission einer idiopathischen plastischen Anämie nach 21 Monaten die für die paroxysmale nächtliche Hämoglobinurie typischen Veränderungen der Erythrocyten auftraten.

Résumé

Rapport sur le cas d'une jeune fille de 19 ans qui après être apparemment remise d'une anémie plastique idiopathique présenta 21 mois plus tard des altérations érythrocytaires typiques pour l'hémoglobinurie nocturne paroxystique.

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Normierung in der Hämatologie

Mitteilungen der Hämometerprüfstelle

Nachdem in den letzten Jahren die Hämoglobinometrie genormt worden ist (DIN 58 931/1–4 Acta haemat. 35, 255 [1966]) ist in den vergangenen Monaten mit der Normung weiterer hämatologischer Routinemethoden begonnen worden; es sind die ersten Normentwürfe über die Blutkörperchenzählung, die Hämatokritbestimmung und die Erythrocytensenkungsreaktion erschienen.

Der Entwurf DIN 58 932, Blatt 1 trägt den Titel «Bestimmung der Anzahl der Blutzellen und -plättchen. Begriffe, Maßeinheiten, Probenahme und Vorbereitung». Die einzelnen Abschnitte heißen 1 Zweck, 2. Begriff, 3 Entnahme von Blutproben, 4. Vorbereiten der Blutprobe 5. Zuverlässigkeitskontrollen, 6. Befundbericht, wonach ausführliche Erläuterungen folgen. Wie aus der Bezeichnung der Abschnitte hervor geht, befaßt sich dieses Normblatt mit jenen Handhabungen, die bei der Bestimmung der Erythrocytenzahl, der Leukocytenzahl und der Blutplättchenzahl vorgenommen werden, unabhängig davon, ob der Zählvorgang selbst dann in herkömmlicher Weise mit der Zählkammer oder wie dies heute in Kliniklaboratorien üblich ist, mit einem Blutkörperchenzählautomaten durchgeführt wird. Es ist beabsichtigt, auch diese Vorgänge zu standardisieren.

Der Entwurf DIN 58 933, Blatt 1 behandelt die «Bestimmung des Zellpackungsvolumens im Blut. Begriffe, Maßeinheiten, Verfahren». Die einzelnen Abschnitte führen die Titel 1 Zweck, 2. Begriffe, 3 Maßeinheiten, 4 Standard-Methode, 5. Geräte, 6. Durchführung der Bestimmung, 7 Auswertung, wonach eine Zusammenstellung des Schrifttums und ausführliche Erläuterungen folgen. Der Ausdruck «Zellpackungsvolumen» (englisch packed cell volume) ist eine im deutschen Sprachgebrauch bisher wenig verwendete Bezeichnung, die eingeführt werden mußte, da neuerdings neben der – auch in diesem Normblatt empfohlenen – Standard-Methode, dem Hämatokrit verfahren, auch andere Methoden (Messung des elektrischen Widerstandes im Vollblut) propagiert werden. Als «Hämatokrit Wert» soll in Zukunft nur das mittels der Hämatokrit-Methode festgestellte Zellpackungsvolumen der roten Blutkörperchen im Vollblut genannt werden. Als Standard-Methode wird der Mikrohämatokrit (in abschmelzbaren Kapillaren) in hochtourigen Zentrifugen mit Spezialkopf (Minimum etwa 10 000 U/min) empfohlen. Auch die Maße der Glaskapillaren und die zu verwendende Glassorte sind in diesem Entwurf beschrieben worden.

Der Entwurf DIN 58 935 endlich heißt «Bestimmung der Senkungsreaktion im Blut. Begriffe, Verfahren». Die Abschnitte tragen die Titel 1 Zweck, 2. Begriff 3. Standard-Methode, 4 Reagenzien und Geräte, 5. Probenahme, 6. Messung, 7 Befund, 8. Zuverlässigkeitskontrollen. Auch hier sind anschließend die wichtigsten Literaturstellen und ausführliche Erläuterungen angegeben. Als Standard-Methode ist die Originalmethode von Westergren empfohlen worden, wie sie ja auch in Deutschland allgemein eingeführt ist. Es wird empfohlen, zur Blutabnahme Spezialspritzen, die bei 0,4 und 2,0 ml einrasten, zu verwenden. Die Schrägstellung der Röhrchen zur «Schnellsenkung» wird abgelehnt. Im Gegenteil, es wird empfohlen, die Gestelle mit einem Lot und einem Thermometer auszurüsten, da die Senkungsreaktion bekanntlich sehr tem-

peratur- und lageempfindlich ist. Es wird empfohlen, einheitlich den Namen «Senkungsreaktion» oder «Erythrozytensenkungsreaktion» zu verwenden, da sich daraus eine internationale Kürzung «ESR» ableiten läßt, was auch gut dem englischen Ausdruck «Erythrocyte Sedimentation Rate» entgegenkommt (die infolge eines Flüchtigkeitsfehlers der Redaktion in den Erläuterungen stehengebliebene Kürzung «BSG» sollte nicht beachtet werden).

Da es sich bei diesen Normentwürfen um grundlegend wichtige Untersuchungsmethoden handelt, die jeder Arzt täglich selbst durchführt oder durchführen läßt, sind alle Kollegen dringend gebeten, sich die Normentwürfe über den Buchhandel (oder direkt beim Beuth-Vertrieb) zu beschaffen und, falls es nötig erscheint, kritisch dazu Stellung zu nehmen. In der nächsten Zeit beabsichtigt der Unterausschuß Hämatologie weitere Blätter dieser Normen auszuarbeiten und auch die hämatologische Nomenklatur zu bearbeiten. Anregungen, Vorschläge, Einwände zur Normungsarbeit sind zu richten an die Hämometerprüfstelle (78 Freiburg, Hugstetterstraße 55) oder direkt an den Deutschen Normenausschuß (1 Berlin 30, Burggrafenstraße 4–7).

K. G. von Boroviczény, Freiburg im Br.

Libri

E. Kleihauer: Fetales Hämoglobin und fetale Erythrocyten. Vergleichende Untersuchungen über strukturelle und funktionelle Besonderheiten der roten Blutzelle Neugeborener und junger Säuglinge. Beiheft zum Archiv für Kinderheilkunde Heft 53. Verlag Ferd. Enke, Stuttgart, 1966. 190 S., 56 Abb., 7 Tab. Preis geheftet DM 52.

Die mit einem Geleitwort von Prof. K. Betke versehene Monographie enthält eine sehr gute und vollständige Zusammenstellung aller heutigen Kenntnisse über Bestandteile und Eigenschaften der fetalen roten Blutkörperchen mit vielen Ergebnissen eigener Forschungen des Autors. Nach einem kurzen Überblick über die verschiedenen normalen menschlichen Hämoglobine werden eingangs die Nachweis- und Bestimmungsmethoden sowie die Eigenschaften des fetalen Hämoglobins behandelt. Dann folgen einige Kapitel über das postnatale Vorkommen von fetalem Hämoglobin unter physiologischen und pathologischen Verhältnissen. Der nächste Teil ist den fetalen Erythrozyten gewidmet, wobei strukturelle und funktionelle Besonderheiten, Alterungsvorgänge und alle wichtigen Stoffwechselmechanismen besprochen werden. Am Schluß werden die Beziehungen zwischen fetalem Hämoglobin und den Eigenschaften der fetalen Erythrozyten behandelt.

Die überaus gut gelungene Monographie ist übersichtlich gegliedert, mit sehr instruktiven Abbildungen, Diagrammen und Tabellen ausgestattet und enthält am Schluß ein ausgedehntes Literaturverzeichnis. Hämatologen, Pädiater und alle, die sich im Laboratorium mit Hämoglobin und Erythrozyten beschäftigen, werden das Heft als wertvolles Nachschlagewerk willkommen heißen.

H. R. Marti, Basel

Acta haemat. 38: 65–78 (1967)

Medizinische Universitätsklinik, Freiburg im Br
(Direktor: Prof. Dr Dr h.c. L. Heilmeyer)

Untersuchungen über die Hämsynthese in roten Blutzellen

II Mitteilung: Die Bildung von Fe^{59}-Hämoglobin in peripheren menschlichen Erythrozyten in vitro*

H. Hempel, W Schmidt** R. Clotten und L. Heilmeyer

Junge kernlose Säugetiererythrozyten können *in vivo* und *in vitro* Hämoglobin bilden, wenn auch in viel geringerem Ausmaß als ihre kernhaltigen Vorstufen im Knochenmark, wie Walsh u. Mitarb (12) erstmals mit Hilfe von radioaktivem Eisen zeigen konnten. In einer vorhergehenden Mitteilung (5) wurde über die Hämoglobinbildung in Kaninchenerythrozyten und ihre vergleichende Messung *in vitro* mit Hilfe der Radioeisenmethode berichtet. Die vorliegende Arbeit beschäftigt sich mit ähnlichen Untersuchungen an Blutproben von normalen Versuchspersonen und von Patienten mit verschiedenen Blutkrankheiten, die zur Entscheidung der Frage durchgeführt wurden, ob sich bei Anämien mit den ferrokinetischen und biochemischen Zeichen einer Eisenverwertungsstörung, also den sideroachrestischen Anämien im weiteren Sinne, die Hämsynthesestörung direkt in den peripheren Erythrozyten nachweisen läßt. Entsprechende Arbeiten von anderer Seite (1 4 2, 7 8 11) haben zu widersprüchlichen Ergebnissen geführt.

Methoden

Die Blutproben wurden aus der Vene mit einer heparinbenetzten Spritze entnommen und sofort in ein Eiswasserbad verbracht. Die Erythrozyten wurden vom Plasma getrennt, dreimal mit eiskalter Kochsalzlösung gewaschen und zur Herstellung der Suspensionen mit Kochsalzlösung, zur Herstellung der Hämolysate mit destilliertem Wasser im Verhältnis 1 2 gemischt. Alle Arbeiten von der Entnahme bis zum Beginn der Inkubation wurden bei 4 °C vorgenommen.

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* Mit Unterstützung der Deutschen Forschungsgemeinschaft.

** Wesentliche Teile wurden aus der Inaugural-Dissertation von W. Schmidt entnommen.

peratur und lageempfindlich ist. Es wird emp... setzung:
reaktion» oder Erythrozytensenkungsrea... 3 ml
internationale Kürzung «ESR» ableite... 2 ml
«Erythrocyte Sedimentation Rate» ... 1 ml
der Redaktion in den Erläuter... 1 ml
achtet werden) 1 ml

Da es sich bei ... wurde, wenn nicht anders angegeben, 1 ml einer
methoden ha... ...ng eingesetzt, da die früher beschriebenen Versuche
sind alle Ko... ...eigt hatten, daß nach Lyse der Zellen eine erfaßbare
direkt beir... ...substratkonzentrationen abläuft. Die Ansätze wurden im
Stellung ... bei 37 °C unter langsamer Rotation inkubiert. Anschließend
weitere ... ...n Zugabe einer eiskalten Trägerhämoglobinlösung gestoppt,
zu be... ...siert und seine Radioaktivität gemessen. Die Technik der Iso-
richte... ...nach der Inkubation durch Häminkristallisation oder Hämoglobin-
an d... in der vorhergehenden Mitteilung (5) eingehend beschrieben. Als
diese wird der Prozentsatz des im Hämin wiedergefundenen Radio-
...auf die insgesamt zugesetzte Radioeisenmenge, angegeben.

Ergebnisse

1. Ermittlung der optimalen Reaktionsbedingungen. Die früher ge-...chte Beobachtung, daß durch Hämolyse die Fähigkeit zur Häm-...bildung stark vermindert wird und nur bei unphysiologisch hohen Substratkonzentrationen wieder in Gang kommt, ließ sich auch an menschlichen Erythrozyten bestätigen (Tab. I). Der optimale pH-Wert lag zwischen 7,3 und 7,5, wie in einem Hämolysatversuch mit verschiedenen Phosphatpuffermischungen festgestellt werden konnte (Abb. 1). Dies entspricht den Angaben von Lochhead *et al.* (6) und unseren Erfahrungen bei Kaninchenretikulozyten. Schwartz *et al.* (9) sowie Yoshikawa und Yoneyama (13) fanden an Vogelerythrozyten dagegen pH-Optima von 7,9 bzw. 8,2.

Die optimale Protoporphyrinkonzentration lag ebenso wie in einem Teil unserer Kaninchenversuche etwa bei 1×10^{-4} M (Abb. 2). Bei Konzentrationen von 5×10^{-4} M kam es bereits zu

Tabelle I
Hämsynthese in den Erythrozyten von zwei Patienten mit erworbener hämolytischer Anämie.

	Pat. I	Pat. II
Retikulozyten ‰	247	375
Fe^{59} Einbau %		
Suspension ohne Substrat	24,1	74,8
Hämolysat ohne Substrat	4,7	1,5
Hämolysat mit Protoporphyrin	25,3	12,4
Hämolysat mit δ-Aminolaevulinsäure	19,2	

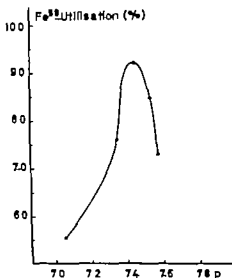


Abb. 1. Abhängigkeit der Hämsynthese vom pH Wert.

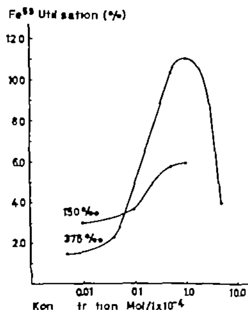


Abb. 2. Abhängigkeit der Hämsynthese von der Substratkonzentration.

einer deutlichen Hemmung der Reaktion. GOLDBERG (3) und VAVRA et al (11) haben etwas niedrigere optimale Porphyrinkonzentrationen von 5×10^{-5} M angegeben.

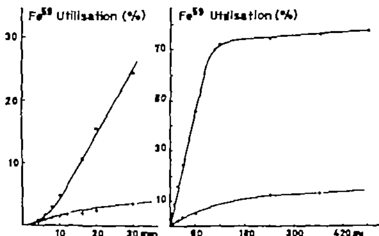


Abb. 3. Zeitlicher Verlauf der Hämsynthese *in vitro*. O = Suspension, • = Hämolysat.

Auch der zeitliche Verlauf der Hämsynthese entspricht etwa den Verhältnissen bei Kaninchenretikulozyten, wie Abb 3 zeigt. In der Mehrzahl der Versuche war der Anstieg des Fe^{59} Einbaus in der ersten Stunde etwa linear nach der vierten Stunde erfolgte nur noch eine geringe zusätzliche Hämsynthese.

2. *Leerwert der Isolierungsmethode und Ergebnisse bei Normalpersonen.* Auch bei sorgfältiger Waschung des rekristallisierten Hämins oder des Hämoglobinpräzipitats bleibt eine geringe Verunreinigung durch radioaktives Nichthämeisen zurück. Um diese zu ermitteln wurde eine Hämoglobinlösung aus längere Zeit gelagerten Konservenerythrozyten hergestellt und mit einer Radioeisenlösung ohne weitere Zusätze gemischt die Mischung wurde sofort in der üblichen Weise verarbeitet und die Radioaktivität im rekristallisierten gewaschenen Hämin bzw im gewaschenen Hämoglobinpräzipitat gemessen. Bei der Kristallisationsmethode (6 Proben) betrug die Verunreinigung $0.12 \pm 0.05\%$ bei der Präzipitationsmethode $0.04 \pm 0.03\%$ der zugesetzten Radioeisenaktivität. Da eine stärkere unspezifische Anlagerung des Eisens an die Proteine der Hämoglobinlösung oder auch eine geringe nicht enzymatische Hämbildung theoretisch vorstellbar ist, wurden außerdem Erythrozyten aus älteren Blutkonserven, die zur sicheren Inaktivierung eventuell noch vorhandener Hämsynthetase mehrere Stunden bei Zimmertemperatur gestanden hatten, in typischer Weise 4 h lang inkubiert. Die scheinbaren Fe^{59} Einbauwerte sind in Tab. II aufgeführt. Man erkennt, daß bei Isolierung des Hämeisens mittels Präzipita-

Tabelle II

Scheinbare Fe^{59} Einbauwerte (%) bei Konservenerythrozyten. Als «Leerwert» wurde der Mittelwert + doppelte Standardabweichung angenommen. Zum Vergleich mit den Einbauwerten bei Patienten wurde auch der Mittelwert beider Methoden angegeben.

	Suspension			Hämolysat		
	Präz.	Krist.	Mittel	Präz.	Krist.	Mittel
Zahl der Proben	14	12	26	13	11	24
Mittelwert	0,10	0,04	0,07	0,17	0,09	0,14
«Leerwert»	0,22	0,06	0,13	0,31	0,17	0,24

Tabelle III

Hämsynthese *in vitro* in peripheren Erythrozyten von gesunden Versuchspersonen.

VP Nr	Geschlecht	Retikulocyten ‰	Fe^{59} Einbau % Suspension Präz.	Suspension Krist.	Hämolysat Präz.	Hämolysat Krist.
1	m	8	0,28	0,30	0,98	0,97
2	m	9	0,05	0,04	0,19	0,58
3	m	9	0,49	—	1,19	—
4	m	16	0,70	—	0,81	—
5	m	10	0,13	0,04	0,26	0,12
6	m	10	0,08	—	0,16	0,11
7	w	10	0,40	0,19	0,61	0,89
8	w	7	0,03	0,14	0,18	0,24
9	w	7	0,00	0,12	0,32	0,29
10	w	18	0,56	0,58	0,22	0,12

tion die Verunreinigung nach 4stündiger Inkubation deutlich höher liegt als bei sofortiger Weiterverarbeitung des Ansatzes dagegen ist bei Verwendung der Kristallisationsmethode kein Unterschied zu erkennen, was gegen eine meßbare nichtenzymatische Hämsynthese unter den verwendeten Bedingungen spricht.

Bei der Mehrzahl der gesunden Versuchspersonen lagen die Fe^{59} Einbauwerte eindeutig über dem Leerwert. (Tab III) bei einigen war dies nur mit Hilfe der bei niedrigen Einbauwerten besser geeigneten Kristallisationsmethode zu erfassen (z. B. Nr 8 und 9) Bei einigen Normalblutproben lagen die Hämsynthesewerte sicher im Bereich des Leerwertes.

3. Ergebnisse bei Patienten mit verschiedenen Blutkrankheiten. Die Ergebnisse bei Blutproben von insgesamt 28 Patienten sind in Tab IV zusammengestellt. Der besseren Übersichtlichkeit halber

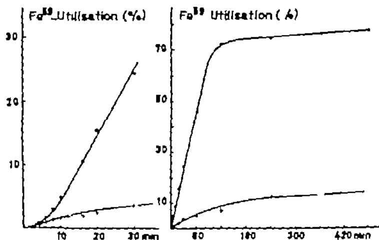


Abb. 3. Zeitlicher Verlauf der Hämsynthese in vitro. O = Suspension, x = Hämolysat.

Auch der zeitliche Verlauf der Hämsynthese entspricht etwa den Verhältnissen bei Kaninchenretikulozyten, wie Abb 3 zeigt. In der Mehrzahl der Versuche war der Anstieg des Fe^{59} Einbaus in der ersten Stunde etwa linear nach der vierten Stunde erfolgte nur noch eine geringe zusätzliche Hämsynthese.

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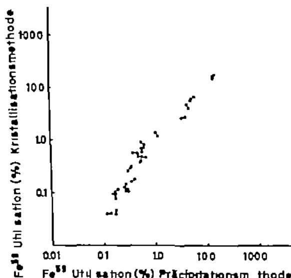


Abb. 4 Vergleich der Ergebnisse der Kristallisations- und der Präcipitationsmethode

Untersuchungen die mit beiden Methoden gewonnenen Werte getrennt angegeben. Die Hämoglobinpräcipitate sind zwar nach 4-stündiger Inkubation der Hämolysate etwas stärker mit freiem Radioeisen verunreinigt als das kristallisierte Hämin, die Unterschiede sind aber so niedrig, daß sie zumindestens bei übernormalen Fe^{59}-Einbauwerten bei erhöhter Retikulocytenzahl vernachlässigt werden können.

Genau wie bei Kaninchenretikulocyten wird auch bei menschlichen Erythrocyten eine noch vorhandene Fähigkeit zur enzymatischen Hämsynthese durch Hämolyse der Zellen weiter vermindert, so daß unphysiologisch hohe Substratkonzentrationen notwendig sind, um überhaupt noch eine meßbare Hämsynthese in Hämolysaten zu erhalten (Tab. I) In Hinsicht auf diese Beobachtung haben wir sowohl Suspensionen intakter Zellen ohne Porphyrinzusatz als auch Hämolysate mit optimaler Protoporphyrinkonzentration inkubiert, um einerseits den physiologischen Verhältnissen möglichst nahezukommen, andererseits den Einfluß einer eventuell vorhandenen Protoporphyrinbildungsstörung auf das Ergebnis auszuschalten.

Bei der Mehrzahl unserer *Normalpersonen* lag die Fe^{59} Hämbildung signifikant über dem Leerwert, der an inaktiven Hämoglobinlösungen bestimmt worden war In der Literatur finden sich diesbezüglich überraschenderweise nur wenige Angaben. Goldberg *et al.* (3) sowie Gaidos und Gaidos (2) konnten bei einigen Gesunden mit Hilfe der auch von uns verwendeten Radioeisenmethode keine Hämbildung nachweisen. Dagegen berichten Najean *et al.* (7 8) über den Nachweis der Hämsynthetase in den Erythrozyten von 16 gesunden Personen. Mindestens ebenso wichtig wie der gelungene Nachweis einer enzymatischen Hämsynthese in nor

malen kernlosen Erythrozyten erscheint uns die Tatsache, daß bei einigen Normalblutproben keine Hämsynthetase nachweisbar war auch wenn nur die Ergebnisse der bei kleineren Einbauwerten geeigneteren Kristallisationsmethode berücksichtigt werden. Wir können damit von vornherein sagen daß eine pathologische Verminderung der Hämsynthetaseaktivität in Blutproben mit normaler Retikulozytenzahl mit Hilfe der Radioeisenmethode nicht sicher nachweisbar sein wird. Da sich bei Patienten mit erhöhter Retikulozytenzahl teilweise sehr hohe Fe^{59} Einbauwerte fanden, haben wir die Korrelation zwischen Retikulozytenzahl und Fe^{59} Hämoglobinbildung geprüft, um auf diese Weise eventuelle Abweichungen vom normalen Verhalten bei Personen mit Eisenverwertungsstörungen zu finden. Wie Abb 5 zeigt, geht die mit der Radioeisenmethode erfaßbare Hämbildung der Retikulozytenzahl zwar richtungsmäßig paralell, zeigt aber beim Vergleich der Blutproben verschiedener Patienten nur eine sehr lose Korrelation.

Auch wenn man den Reifungsgrad der einzelnen Retikulozytenformen mit berücksichtigt, kommt man zu etwa gleichen Ergebnissen (Abb 6) Aus der beschriebenen Korrelation zwischen Fe^{59} Einbau und dem an Hand der Retikulozytenzahl geschätzten Altersaufbau der Erythrozytenprobe ergibt sich in unserem Unter

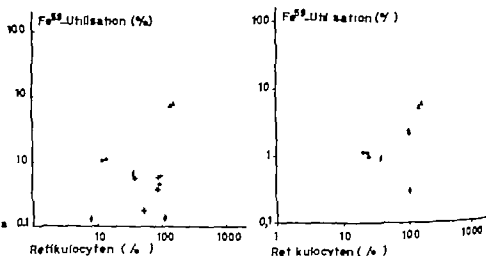


Abb. 5. Abhängigkeit des Fe^{59} Einbaus von der Retikulozytenzahl (a Suspension, b Hämolysate).
+ hämolytische Anämie, # Thalassämie, ♦ sideroachrestische Anämie, ♦ perniciöse Anämie, ▲ Osteomyelofibrose, ○ Eisenmangelanämie, ● Panmyelopathie, □ akute Blutungsanämie, ■ Sichelzellenanämie, ✱ Morbus Gierke.

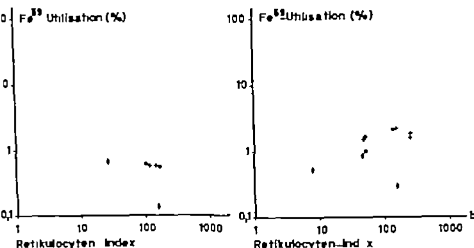


Abb. 6. Abhängigkeit des Fe^{59}-Einbaus von der Retikulozytenzahl und vom mittleren Reifungsgrad der Retikulozyten (a Suspensionen, b Hämolysate). Bedeutung der Symbole: siehe Abb. 5.

suchungsmaterial kein typisches Verhalten bestimmter Erkrankungsgruppen, insbesondere keine verminderte Fe^{59}-Hämbildung bei allen Patienten mit dem klinisch-hämatologischen Zeichen einer Eisenverwertungsstörung. Eine auffällig niedrige Hämsynthese zeigten nur die Erythrozyten von Fall 5 (hämolytische Anämie mit Eisenverwertungsstörung, 64‰ Retikulozyten*) und von Fall 25 (Thalassaemia major, 52‰ Retikulozyten). Zu ähnlich uneinheitlichen Ergebnissen kamen auch VAVRA *et al.* (11), die bei 10 Patienten mit Thalassämie eine normale Hämbildung fanden, sowie BANNERMAN *et al.* (1), die zwar neben einer bewiesenen Aktivitätsverminderung der δ-Ala-dehydrogenase eine Störung des letzten Schrittes der Blutfarbstoffsynthese aufgrund der in Thalassämieerythrozyten erhöhten Protoporphyrinkonzentrationen vermuteten, dies aber bei 4 Patienten mit Hilfe von Radioeisen und auch von C^{14}-Protoporphyrin nicht nachweisen konnten. Wichtig erscheint in diesem Zusammenhang, daß bei uns die Fe^{59}-Hämbildung weder im Hämolysat (bei unphysiologisch hoher Protoporphyrinkonzentration) noch in der Suspension (bei normaler Protoporphyrinkonzentration) regelmäßig eine signifikante Verminderung erkennen ließ. Damit wird auch die von BANNERMAN *et al.* (1) aufgestellte Hilfs-

* Inzwischen haben Familien-Untersuchungen hier den Verdacht auf das Vorliegen einer Thalassämie ergeben.

hypothese, daß der postulierte Hämsynthetasemangel durch die hohe Substratkonzentration im Ansatz verdeckt würde, entkräftet. Im Gegensatz zu diesen genannten Ergebnissen fanden französische Autoren (2 7 8) bei Thalassämien und sideroachrestischen Anämien eher *erhöhte* Hämsynthesewerte. Aus methodischen Gründen können die Zahlenwerte dieser Untersuchungen nicht mit unseren eigenen verglichen werden. Von GAIDOS werden die Ergebnisse bei 25 Patienten mit den verschiedensten Blutkrankheiten mit 4 Normalen verglichen, die keine Hämsynthese *in vitro* zeigten. Die von NAJEAN angegebenen Werte wurden auf täglich mitgeführte Normalkontrollen bezogen die relativ starke Schwankungen aufwiesen.

An Hand unserer Beobachtungen, die ebenso wie die von VAVRA die große Variabilität der Hämsynthetaseaktivität innerhalb einer Krankheitsgruppe deutlich machen, erscheinen uns die Angaben von GAIDOS und von NAJEAN über eine Erhöhung der Hämbildung bei Eisenverwertungsstörungen, die ja auch theoretisch kaum verständlich wäre, nicht bewiesen zu sein.

Die Schwankungen des prozentualen Fe^{59} Einbaus bei altersmäßig vergleichbaren Blutproben sind teilweise auf einen verschieden großen Vorrat der Erythrozyten an intrazellulärem hämsynthesefähigem Eisen zurückzuführen, wie VAVRA (11) durch vergleichende Untersuchung der Hämsynthese mit verschiedenen markierten Substraten zeigen konnte. Aus einer größeren Menge freien intrazellulären Eisens resultiert eine stärkere Verdünnung des zugesetzten Radioeisens, so daß sich allein durch die verminderte spezifische Radioaktivität des in die Reaktion eingehenden Fe^{59} ein verminderter prozentualer Fe^{59} Einbauwert ergeben kann. Wir haben deswegen auf die unsichere Mengenangabe des *in vitro* gebildeten Häms verzichtet.

Aufgrund unserer Ergebnisse müssen wir sagen, daß die Untersuchung der Fe^{59} Hämbildung in peripheren Erythrozyten allein nicht geeignet ist, die Frage einer Störung der Protoporphyrin-Eisenverbindung insbesondere die Frage eines Hämsynthetasemangels als naheliegende Ursache der sideroachrestischen Anämien zu entscheiden. Dafür sind teilweise methodische Gründe verantwortlich, die durch die Verwendung markierter Porphyrine an Stelle von Radioeisen zu umgehen wären. Darüber hinaus scheint aber die physiologisch bedeutungslose Hämsynthetase-Restaktivität in den peripheren Erythrozyten kein sicheres Maß für die Hämsynthetaseaktivität in den kernlosen roten Blutzellen im Knochen-

mark zu sein. Weitere Untersuchungen der Hämsynthese mit Fe^{59} in kernlosen roten Blutzellen scheinen deswegen wenig sinnvoll. Die Prüfung der Hämsynthese an Erythroblasten aus menschlichem Knochenmark, über die bisher nur STEINER (10) berichtet hat, soll Gegenstand einer folgenden Mitteilung sein.

Zusammenfassung

Die enzymatische Blutfarbstoffbildung aus Eisen und Protoporphyrin in peripheren menschlichen Erythrocyten wurde durch Inkubation von Hämolysaten und Zellsuspensionen mit Protoporphyrin und Radioeisen untersucht. Die höchsten Synthesewerte zeigen Blutproben mit hohem Retikulocytengehalt, ohne daß zwischen der Retikulocytenzahl und der Menge des in vitro gebildeten Fe^{59} Hämoglobins eine feste Beziehung besteht. Auch in Normalblutproben läßt sich meist noch eine geringe Hämsynthetaseaktivität nachweisen. Bei Thalassämien und sideroachrestischen Anämien konnte nur in Einzelfällen eine charakteristische Veränderung der Fe^{59}-Hämoglobinbildung in vitro gegenüber altersmäßig vergleichbaren Blutproben von Normalpersonen oder von Patienten mit Hämolyse oder Blutung nachgewiesen werden.

Summary

Enzymatic formation of blood pigments from iron and protoporphyrin was studied in human peripheral erythrocytes by means of incubation of haemolysates and cell suspensions with protoporphyrin and radio-iron. Samples with high reticulocyte counts show the highest rate of synthesis, although there is no fixed relationship between the number of the reticulocytes and the amount of ^{59}F haemoglobin formed in vitro. A limited degree of haem synthetase activity can usually also be seen in normal blood samples. In thalassaemia and iron-deficiency anaemia characteristic alteration in ^{59}F haemoglobin formation compared with samples from normal persons of the same age or patients with haemolysis or haemorrhage was seen only in isolated cases.

Résumé

La synthèse enzymatique érythrocytaire du hème à partir du fer et de la protoporphyrine été étudiée par l'incubation d'hémolysats et de suspensions de cellules avec de la protoporphyrine et du fer radio-actif. C'est dans les échantillons sanguins ayant la plus forte concentration de réticulocytes que la synthèse fut la plus active sans que n exista pourtant une relation constante entre le nombre de réticulocytes et la quantité de Fe^{59} hème formé in vitro. Une légère activité de la synthétase du hème peut être démontrée dans le sang normal. Dans les thalassémies et les anémies sidéro-achrestiques, une modification caractéristique de la synthèse de la F^{59}-hémoglobine in vitro ne put être mise en évidence que dans peu de cas isolés par rapport au sang de personnes de même âge, saines ou souffrant d'hémolyse ou de pertes de sang.

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(22) and MITCHELL (21) using tritiated uridine and leucine. Autoradiographic investigations using DNA and RNA precursors as well as labelled aminoacid have been carried out by TORELLI et al (37) in normal human plasma cells and their precursors in one case of reactive plasmocytosis. Autoradiographic studies on myeloma cells have so far been restricted to the use of tritiated thymidine (18, 14) in order to evaluate their proliferative activity The results obtained have confirmed the low mitotic activity of these cells, which had previously been established by the stathmokinetic test (17)

In the present study in order to extend these preliminary autoradiographic observations and obtain a more comprehensive picture of the cytochemical pattern of normal and myelomatous plasma cells, additional radioisotopes were employed, as well as a broad spectrum of cytochemical reactions.

Material and Methods

The total number of cases t our disposal consisted of 12 cases of multiple myeloma, 2 cases of plasma cell leukaemia, one case of reactive plasmacytosis and 10 cases with no malignant haematological disorder but with adequate numbers of bone-marrow plasma cells. Cytochemical tests were performed on all cases, while autoradiographic investigations were carried out on more restricted number of cases.

The cytochemical techniques used in this study consisted of the azo-dye method for alkaline phosphatase, described by HAYHOE and QUAGLINO (10) the technique of ROZENSZAJN et al. (33) for acid phosphatase, the peroxidase reaction, modified by QUAGLINO and FLEMANS (31) the Sudan black B reaction for staining lipids (35) the acid hematein method of BAKER (1) for showing phospholipids, the periodic acid Schiff reaction (19) for staining polysaccharides, the technique described by WACHSTEIN and WOLF (38) for demonstrating non-specific esterases, and staining methods for demonstrating succinic and DPN-linked dehydrogenases (28, 32) and finally cytochrome oxidase, modified by QUAGLINO et al. (30).

For the study of DNA, RNA and protein metabolism, thymidine, cytidine and uridine were used at concentration of 5 $\mu c/ml$, while leucine was employed at concentration of 10 $\mu c/ml$. Autoradiograms were prepared using either Kodak AR 10 stripping films or NTB 2 emulsion. The contact period varied according to the isotope used 6 days for tritiated thymidine, 6 days for tritiated leucine, and 10–30 days for uridine-5-T and tritiated cytidine. In order to evaluate the metabolic stability of RNA (37) actinomycin D, at concentration of 10 $\mu g/ml$, was added to the incubation medium.

Results

Cytochemical Findings

The cytochemical investigations were carried out on the bone-marrow preparations of the cases previously listed and the results for each different staining method have been described separately

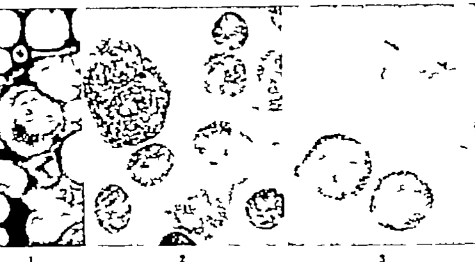


Fig 1 Normal plasma cell showing faint diffuse positivity with Baker acid hematein reaction.

Fig 2. Periodic acid Schiff (PAS) reaction in case of multiple myeloma. While the majority of plasma cells are negative or show only faint diffuse positivity the single binucleated myeloma cell contains numerous PAS positive cytoplasmic granules.

Fig 3. A case of plasma cell leukaemia showing two plasmoblasts with numerous coarse PAS positive granules in the cytoplasm and three more mature myeloma cells, displaying diffuse cytoplasmic positivity

Lipids Normal plasma cells showed a negative Sudan black B reaction and a faint diffuse positivity with Baker's acid hematein technique, visible only in uncounterstained preparations (Fig 1) Multiple myeloma cells showed the same features, although in one case of plasma cell leukaemia, a small proportion of the atypical plasmoblasts showed a few sudanophilic granules, which were better visualized in the absence of any counterstain.

PAS reaction. The greatest individual variability was seen when plasma cells were subjected to this staining reaction. While in non-malignant conditions, plasma cells were devoid of any granular positivity and showed only weak diffuse staining in multiple myeloma there appeared to be a tendency for PAS positivity to increase in those cases in which there was an increased number of immature or atypical plasma cells (Fig 2) Indeed the most striking accumulation of PAS positivity was observed in the two cases of plasma cell leukaemia (Fig 3)

Diastase or salivary amylase digestion in normal plasma cells and in all pathological cases virtually abolished both diffuse and



4 5

Fig 4 Two fields from an typical case of multiple myeloma, in which plasma cells show multi-shaped bodies and crystal-like inclusions. MGG stain.

Fig 5. The same case as in Fig 4 after PAS stain. Numerous PAS positive granules are visible t the periphery of the cytoplasm in an early plasma cell precursor the inclusions present in the other myeloma cell, are intensely stained by the PAS reaction.

granular cytoplasmic positivity with the exception however of two cases of multiple myeloma, one in which plasma cells contained Russel bodies and the other in which plasmocytes were covered by peculiar inclusions and crystals (Fig 4 and 5) In this single case additional cytochemical investigations showed that the inclusions were most probably related to glycoproteins since reactions for metachromasia such as azur A and toluidine blue became positive only after sulphation, the alcian blue reaction was negative and fast green at pH 1 2 was moderately intense.

Peroxidase reaction. This enzyme was constantly absent from plasma cells at all stages of maturation, whether normal or ma lignant

Alkaline and acid phosphatase reactions While no alkaline phosphatase positivity either nuclear or cytoplasmic was observed in any plasma cell, a positive cytoplasmic reaction in the form of fine granules, occasionally associated with a diffuse tinge was seen with the acid phosphatase technique. In multiple myeloma a higher percentage of cells showed acid phosphatase reactions and the positivity was more intense although ample individual variations could be detected (Fig 6)



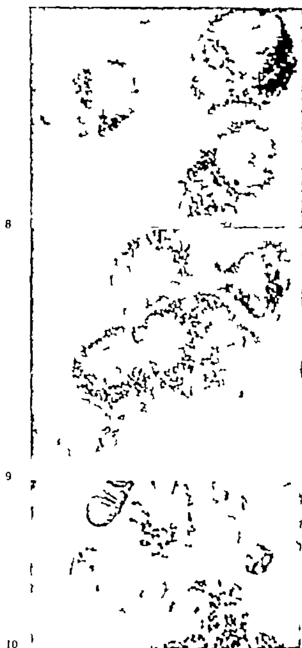
Fig 6 Group of myeloma cells showing predominantly granular positivity with the acid phosphatase reaction.

Fig 7 Group of 3 plasma cells from case of multiple myeloma showing intense non-specific esterase activity in large area of the cytoplasm.

Non specific esterases Non-specific esterases in the form of a scattering of fine bluish granules were observed in the cytoplasm of almost all normal plasma cells. In myelomatous plasma cells esterase activity appeared greatly increased and positivity was present in the form of tightly packed and rather coarse granules (Fig 7)

Succinic and DPN linked dehydrogenases Very strong cytoplasmic reactions mostly granular were obtained using the two substrates sodium succinate and sodium lactate although the intensity of the reaction was greater with the latter (Fig 8 and 9) In one case of plasma cell leukaemia a weak granular reaction was observed with the two substrates in all plasmoblasts both in those with normal or atypical morphology

The observations of WATTENBERG (39) that the lack of available quinone in some rapidly proliferating tumours as well as in various malignancies may be implicated in the observed low rate of succinate oxidation, induced us to try the effect of adding a quinone to the incubation mixture containing sodium succinate and Nitro-BT in order to observe whether myeloma cells showed



a greater increase in the intensity of the staining reaction as compared with normal plasma cells

Menadione at a concentration of 0.2 mg/ml caused increased enzyme activity in both normal and myelomatous plasma cells, but

the enhancement in the latter did not appear to be greater than in normal plasma cells.

Cytochrome oxidase Normal plasma cells at all stages of maturation showed numerous and rather coarse dark blue granules scattered fairly evenly throughout the cytoplasm Myeloma cells were similarly characterized by intense granular positivity which did not differ in its distribution from that observed in normal plasma cells (Fig 10) In some cases of multiple myeloma the atypical plasma cells showed increased cytochrome oxidase activity as revealed by the greater number and size of the dye deposited in the cytoplasm

Autoradiographic Investigations

These were carried out in one case of reactive plasmacytosis, 8 cases of multiple myeloma and one case of plasma cell leukaemia. The data obtained in the different stages of maturation of the plasma cell series in the case of reactive plasmacytosis have already been illustrated in a previous paper (37) It may be recalled that in normal plasma cells RNA synthesis decreases with increasing maturation, while protein synthesis reaches its greatest intensity only in the more mature cell types. Also thymidine incorporation gradually decreases as maturation progresses. Consequently mature plasma cells show no thymidine uptake, minimal or no incorporation of RNA precursors and very intense labelling with radioactive aminoacid. Table I shows the findings obtained with thymidine leucine and uridine 5-T in the pathological cases studied.

Earlier attempts to subdivide plasma cells into the three different groups (plasmoblasts, proplasmocytes and plasmocytes) identified in the case of reactive plasma cell hyperplasia, were subsequently abandoned, because of the presence of frequent and sometimes conspicuous morphological abnormalities, which prevented any objective classification of the cells under study

From Table I it is apparent that thymidine incorporation was low averaging 5 and never exceeding 10 It is worthwhile pointing out that in the few cells showing thymidine uptake, the

Fig 8. Succinic dehydrogenase activity in three myeloma cells, one of which is binucleated The positivity is predominantly granular and is localized in the cytoplasm The incubation medium contained menadione

Fig 9 Strong granular positivity in group of myeloma cells, including binucleated plasma cell, using sodium lactate substrate DPN and Nitro-BT

Fig 10 Three plasma cells from case of multiple myeloma showing intense granular positivity after staining for cytochrome-oxidase

Table I

The average grain counts were obtained by examining at least 100 cells. Since considerable individual cell variation in the intensity of labelling was observed and since the average grain counts differed widely from case to case, the autoradiographic findings were not subjected to statistical analysis.

Case Number	Thymidine	Leucine		Uridine			
	Percent cells labelled	Mean grain counts	Percent cells labelled	Before actinomycin		1 h after actinomycin	
				Mean grain counts	Percent cells labelled	Mean grain counts	Percent cells labelled
Multiple Myeloma							
Case 1	3	187	100	4 63	64	3.82	63
Case 2	10	93	100	4 18	97	1 07	46
Case 3	2	41	65	3.13	70	0.36	17
Case 4	8	33	46	9.24	34	9	48
Case 5	6.5	31	100	27 0	99	—	—
Case 6	6	30	100	16.12	96	—	—
Case 7	3.5	72.5	98	7 69	82	4.87	70
Case 8	4	134	100	11.3	94	10 7	96
Plasma cell leukaemia							
Case 1	6	12 4	100	26 0	100	—	—

degree of labelling was generally high and in no ways dissimilar from that observed in normal plasma cell precursors

Plasma cells from two cases of multiple myeloma were cultured for a period of 72 h in autologous serum and culture medium TC 199 to investigate whether *in vitro* these cells showed enhanced thymidine incorporation with progression of the culture, as observed in some cases of acute leukaemia (29) This was never the case and the percentage of thymidine labelled cells gradually fell with the ageing of the culture.

With regard to leucine uptake, it is clear from the table that the rate of protein synthesis varied greatly in the different cases examined Generally aminoacid incorporation was lower than that observed not only in the fully mature plasma cells but also in the earlier maturation stages. Only in two cases the rate of protein synthesis seemed to reach levels approaching those found in normal mature plasma cells. Finally it seems worth pointing out that, while in the majority of cases all cells were labelled with leucine in one case about 50 of the cells did not show any evidence of uptake with the radioactive protein precursor

Similarly to leucine uridine incorporation was generally low although there was a rather wide range of variations from case to case. This is at variance with the findings obtained in the earliest

stages of the normal plasma cell series, where the uptake of RNA precursors was high (37)

With regard to the metabolic features of the RNA synthesized, even if our data are somewhat restricted it is apparent from the table that the behaviour was not uniform. In fact in three cases the addition of actinomycin D to the incubation medium did not cause any appreciable decrease in the mean gram counts, while in three other cases uridine incorporation was significantly reduced after one hour incubation with actinomycin D

Discussion

The cytochemical investigations show that normal plasma cells contain little or no glycogen, that only minute amounts of lipids, probably phospholipids, are present (16) and that normally intense enzymatic activity as shown by the technique for demonstrating dehydrogenases, cytochrome-oxidase, acid phosphatase and non-specific esterases is a characteristic feature of these cells.

Compared with normal plasma cells, multiple myeloma cells and even more the primitive cells of two cases of plasma cell leukaemia showed increased quantities of PAS positive material, which in most cases appeared to consist of glycogen. These findings would be in agreement with the results of GIBB and STOWELL (8) and STORTI *et al* (36) who reported increased amounts of glycogen in multiple myeloma and of FERRARA (7) who described the presence of a strong PAS positive reaction in the immature cells of a case of plasma cell leukaemia. Thus the most striking cytochemical finding is the increase in PAS positivity which takes place either in association with increasing morphological abnormalities or in relation to progressive cellular immaturity and lack of differentiation as in plasma cell leukaemia.

The results obtained following salivary amylase digestion indicate that in most cases enzymatic hydrolysis greatly reduces or abolishes PAS positivity a finding which tallies with the observations of STORTI *et al* (36) However it is also apparent from the results reported that in two cases in which Russel bodies and other inclusions were present in plasma cells, the intense PAS positivity of these bodies did not disappear following salivary digestion and was probably related to the presence of glycoproteins. These inclusions have therefore a completely different chemical composition

and significance from the vacuolar formations described by MOTT (23) and studied cytochemically by ZLOTNICK (40) and more recently by DI GUGLIELMO (5) which appear to consist mainly of proteins, since reactions for carbohydrates and lipids are completely negative.

With regard to their enzymatic content myeloma cells showed a pattern similar to that of normal plasma cells, although compared with the latter some enzymes (especially acid phosphatase and non specific esterases) were noticeably increased

These findings appear to conform with the results obtained by LOEFFLER and SCHUBERT (15) who studied the activities of acid phosphatase, phosphatase active at pH 7.2 alkaline phosphatase, non specific esterases and amino-peptidases in 200 patients with various diseases and in 38 patients with plasmocytomas. Normal plasma cells were found to have a distinct ATPase activity a moderate acid phosphatase and a weak esterase activity while all other enzymes studied were absent. Myeloma cells showed a higher acid phosphatase and non specific esterase activity than normal, whereas ATPase was reduced or completely absent.

The results of the enzyme studies here reported require some comments regarding the significance of some of the staining patterns observed

The intense positivity obtained in plasma cells both normal and pathological with the dehydrogenase reactions agrees with the histochemical findings of MELDOLESI (20) who also observed that plasma cells showed by far the greatest dehydrogenase activity in the presence of various substrates, compared to all other types of blood cells. However more precise interpretation of the significance of the positivity obtained with DPN (or TPN-) linked dehydrogenases requires extreme caution, because as yet has no means of assessing and controlling the influence of environmental factors and of fixation, the concentration of endogenous pyridine coenzymes and the activity of related diaphorases (6) even in the presence of control smears obtained incubating cells without specific substrates.

Perhaps the results obtained with sodium succinate are the least unreliable because the activity of this enzyme does not require the presence of flavoprotein coenzyme and Nitro-BT reduction may therefore be regarded as the expression of true succinic dehydrogenase activity The addition of menadione does enhance the reaction, but the increased activity in normal plasma cells is in no way greater than in myelomatous ones, finding which appears dissimilar to that found rapidly proliferating tumours

It therefore follows that the high rate of cytochrome oxidase activity present in normal and myelomatous plasma cells is not unexpected in view of the intense activity of succinic dehydrogenase which, together with cytochrome oxidase, belongs to the succinic oxidase system (11) Thus normal plasma cells and also myeloma cells have very active aerobic oxidative systems, which topographically appears to be mitochondrial.

The intense activity shown by the reactions for acid phosphatase and non-specific esterase is less easy to interpret, owing to the greater uncertainty regarding the specific role of these enzymes and, as far as the esterases are concerned, to the low substrate specificity of the latter. Both appear to be localized on lysosomes and therefore an interpretation of the significance of both reactions will only be possible when the function of these cytoplasmic constituents will be better understood.

In conclusion, the results of all these cytochemical studies indicate that the enzymatic pattern of myeloma cells, excepting occasional quantitative variations, is very close to that of normal plasma cells. However on account of the difficulty of distinguishing in myeloma cells stages of differentiation comparable to those which may be observed in the normal plasma cell sequence, the data obtained in multiple myeloma should be evaluated on the basis of the general pattern of metabolic changes occurring in the normal plasma cell series rather than by considering individually the results pertaining to any particular stage of maturation. Such an approach appears particularly convenient when examining the autoradiographic results.

For instance, since myeloma cells show cytochemical characteristics akin to those of maturating plasmocytes, it seems quite natural that these cells should display a low percentage of thymidine incorporation. However the average labelling intensity of myeloma cells does not appear to be comparable to that of cells which are already half way through the differentiation process, since we have previously shown (18) that the rate of DNA synthesis decreases as maturation progresses. On the contrary as mentioned in the results in the small percentage of labelled myeloma cells the rate of thymidine incorporation is high and this suggests that these cells go through the S period at a rate similar to that of normal plasmoblasts. These findings could therefore indicate the probable existence in multiple myeloma of two cell populations one corresponding to a minority of cells, characterized by a normal rate of reduplication and capable of division despite the accumulation in the cytoplasm of metabolic constituents appropriate to more advanced stages of the plasma cell line the second comprising the great majority of cells, apparently devoid of any capacity to proliferate. Such an interpretation is also supported by KILLMANN *et al* (14) although the authors exert some caution in drawing definite conclusions on account of the limitations of the autoradiographic technique in the study of cell kinetics.

A further difficulty in the autoradiographic study of myeloma cell kinetics, especially with regard to the possible existence of a non-dividing cell compartment, is provided by the existence of a variable proportion of polyploid cells, as shown by the cytophotometric studies of PETRAKIS and FOLSTAD (27)

With regard to the results obtained with tritiated leucine, it appears that myeloma cells are unable to reach and maintain a high rate of protein synthetic activity such as usually shown by normal plasmocytes It was previously suggested (37) that in normal plasma cells the intense rate of protein synthesis depends on the formation of messenger RNAs, which are mainly elaborated in the more immature stages.

In myeloma cells the disproportion between the intense enzymatic activity observed and the reduced but persistent protein synthetic activity might be related to a defective elaboration of messenger RNA fractions. Such a contention receives some support from the studies carried out by KIDSON and KIRBY (13) in an experimental differentiated tumour (mouse hepatoma) The authors showed that in the malignant cells examined there was a progressive alteration in the countercurrent distribution profiles of messenger RNA with progression of the tumour The authors suggested that less of the tumour genome was transmitted into messenger RNA

A quantitative and/or qualitative defect in the formation of messenger RNAs is also in keeping with the observed presence of nuclear-cytoplasmic asynchronisms, of developmental arrests at varying stages of maturation and of nuclear nucleolar and cytoplasmic deformities demonstrated by the ultrastructural studies of BESSIS *et al* (2)

Since a complete arrest in RNA synthesis, such as occurs in the normal plasma cell series, seems to be conditioned by a certain sequence in the synthetic processes, it follows that if there exists a disordered and unbalanced production of messenger RNAs, this arrest may not occur Such might be the case with myeloma cells, in which autoradiography shows a persistence of varying degrees of RNA synthesis in a high proportion of the abnormal cell population.

The presence of a variable degree of RNA metabolic instability in myeloma cells, as shown by the use of actinomycin D may be interpreted in several ways, but the more likely explanation is that

this finding reflects the different stage reached in each case by the myeloma cells in their abnormal developmental scale. In this context it is worth pointing out that cases 1 and 8, which showed a fairly stable RNA, were those with the highest leucine grain counts, suggesting that in both cases myeloma cells had reached a fairly advanced stage in the maturation process.

In conclusion, with the cytochemical and autoradiographic techniques used in this study myeloma cells were found to be characterized in the series of cases examined by a different level of differentiation, which can probably be ascribed to the variable degree of alteration in messenger RNA production

Summary

Cytochemically normal and myelomatous plasma cells show negative peroxidase reaction, contain minute amounts of lipids, probably phospholipids and display an intense enzymatic activity when subjected to staining reactions for demonstrating dehydrogenases, cytochrome oxidase, acid phosphatase and non-specific esterase. Compared with normal plasma cells, multiple myeloma cells and the blast cells of two cases of plasma cell leukaemia show increased quantities of intracellular glycogen. With labelled DNA and RNA precursors and radioactive aminoacid, normal plasma cells show decreasing DNA and RNA synthesis with progression of maturation, while protein synthesis becomes maximal in the more mature cell types. In multiple myeloma, thymidine incorporation is present in only a small percentage of cells, but their labelling intensity is generally high. Both leucine and uridine incorporation are low although there is a wide range of variation from case to case. Myeloma cells also show a variable degree of RNA metabolic instability after treatment with actinomycin D The authors conclude that myeloma cells appear to be characterized by quantitative and/or qualitative defects in the formation of messenger RNAs, which may be responsible for the different level of differentiation reached in each case by the abnormal cell population

Zusammenfassung

Normale Plasmazellen und Myelomzellen zeigen zytochemisch eine negative Peroxydasereaktion, sie enthalten kleine Mengen von Lipiden, wahrscheinlich Phospholipiden, und zeigen eine intensive enzymatische Aktivität bei Färbemethoden für Dehydrogenasen, Zytochromoxydase, saure Phosphatase und unspezifische Esterase. Im Vergleich mit normalen Plasmazellen zeigen Myelomzellen und Blasten zweier Fälle von Plasmazellenleukämie gesteigerte Mengen an intrazellulärem Glykogen. Mit markierten DNA- und RNA-Vorstufen und mit radioaktiven Aminosäuren zeigen normale Plasmazellen eine Abnahme der DNA- und RNA-Synthese bei zunehmender Reifung, während die Proteinsynthese ihr Maximum in den reiferen Zellen erreicht. Beim multiplen Myelom findet eine Thymidininkorporation nur in einem kleinen Prozentsatz der Zellen statt, aber die Intensität der Markierung ist im allgemeinen hoch. Die Inkorporation von Leucin und Uridin ist gering und zeigt große Schwankungen von Fall zu Fall. Nach Behandlung mit Actinomycin D weisen Myelomzellen ein verschiedenes Ausmaß der Instabilität des RNA-Stoffwechsels auf. Myelomzellen scheinen durch quantitative oder qualitative Störungen der Bildung von Messenger RNA charakteri-

siert zu sein, die verantwortlich sind fur den verschiedenen Differenzierungsgrad der abnormen Zellpopulation in jedem Fall.

Résumé

Les plasmocytes normaux et les cellules myélomateuses présentent aux examens cytochimiques une réaction négative de la peroxydase. Ils contiennent de petites quantités de lipides, probablement des phospholipides, et ont une intense activité enzymatique, comme le montrent les méthodes tinctoriales employées pour mettre en évidence les déshydrogénases, la cytochrome-oxydase, la phosphatase acide et l'estérase non-spécifique. En comparaison aux plasmocytes normaux, les cellules myélomateuses et les blastes de deux cas de leucémie à plasmocytes contiennent de plus grandes quantités de glycogène. A l'aide de précurseurs radio-marqués d'ADN et d'ARN ainsi que d'amino-acides radio-actifs, il est possible de démontrer que les plasmocytes normaux synthétisent moins d'ADN et d'ARN en devenant plus matures, la synthèse des protéines atteignant son maximum qu'à maturation achevée. Dans le myélome multiple, l'incorporation de thymidine bien que dans un petit pourcentage des cellules, mais l'intensité du marquage est en général grande. L'incorporation de leucine et d'uridine est faible et montre de grandes variations d'un cas à l'autre. Après avoir été traitées à l'actinomycine, les cellules myélomateuses présentent une instabilité variable de l'ARN synthétisé. Les cellules myélomateuses semblent être caractérisées par une perturbation qualitative et quantitative de la formation de l'ARN messager perturbation qui est responsable dans chaque cas des différents degrés de différenciation des populations de cellules anormales.

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Erhöhte Aggregation der Thrombozyten bei essentieller Hyperlipämie

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Die Zunahme thrombotischer coronarer Gefäßverschlüße hat zu zahlreichen Untersuchungen Anlaß gegeben, die sich einerseits mit der Möglichkeit einer erhöhten Tendenz zur Thrombusbildung, andererseits mit der Möglichkeit einer Störung des Fettstoffwechsels bei diesen Erkrankungen beschäftigen. Als erste haben McDonald *et al.* (18) über eine gesteigerte «Klebrigkeit» der Thrombozyten gemessen mit der Methode nach Wright (28, 29) bei coronarsklerotischen Beschwerden berichtet. Ihre Untersuchungen wurden unter anderem von Mustard *et al.* (19) und Slack *et al.* (25) bestätigt. Bredden (8) fand morphologisch eine vermehrte Aggregation der Thrombozyten bei und nach einem Herzinfarkt. Albrink *et al.* (2) sowie zahlreiche andere Arbeitsgruppen haben bei Patienten mit Coronarthrombose erhöhte Triglyzeridwerte im Serum beobachtet (siehe 6).

Da eine Beziehung sowohl einer gesteigerten Thrombozytenadhäsivität oder Thrombozytenaggregation als auch einer Hypertriglyzeridämie zur Coronarthrombose wahrscheinlich ist, erhebt sich die Frage, ob zwischen diesen beiden Faktoren eine Beziehung besteht, das heißt, ob hypertriglyzeridämisches Plasma zu einer erhöhten Thrombozytenadhäsivität oder -aggregation führt. In der vorliegenden Arbeit wurde die Möglichkeit einer Beziehung zur Thrombozytenaggregation zunächst an einem Modellfall, nämlich an Patienten mit essentieller Hyperlipämie, das heißt extrem erhöhten Triglyzeridwerten im Plasma, im Vergleich zu einer Gruppe von Normalpersonen untersucht.

1 In den folgenden Ausführungen wird zwischen den Begriffen Adhäsion, Aggregation und Agglutination unterschieden. Unter der Adhäsion verstehen wir die Fähigkeit der Plättchen, an bestimmten Oberflächen zu haften, Pseudopodien auszubilden

und sich über weite Flächen auszubreiten. Bei der Aggregation lagern sich die Thrombozyten zunächst ohne Strukturverlust aneinander, um später miteinander zu verschmelzen. Die Agglutination ist ein immunologischer Vorgang, der zur Zerstörung der Plättchen führt.

Die Prüfung der Adhäsivität und Aggregation der Thrombozyten, die dem Gerinnungsvorgang vorangehen bzw. ihn einleiten, ist methodisch schwierig. Es wurden unterschiedliche Verfahren ausgearbeitet, die sich in drei Gruppen gliedern.

1. Bei der gebräuchlichsten Methode werden die Thrombozyten durch Glaskontakt zur Desintegration gebracht. Ihre Zahl wird vor und nach diesem Kontakt bestimmt. Der dadurch bedingte Thrombozytenverlust stellt ein Maß für ihre Adhäsions- und Aggregationsfähigkeit dar. Dies erfolgt bei der Methode nach WRIGHT (28, 29) durch Rotation einer Glaskammer, die mit Blut gefüllt ist; nach HELLEM (14) passiert Citratblut durch ein Glasperlenfilter. SALZMAN (22) läßt Nativblut unter Luftabschluß direkt aus der Vene durch ein Glasperlenfilter treten und bestimmt vor und nach Passage dieses Filters die Thrombozytenzahl. Unter den bestehenden scheint dies die beste Methode zur Erfassung der Thrombozytenadhäsivität zu sein.

2. Bei der Methode nach BREDDIN (6), modifiziert nach MARX (17), wird die Ausbreitungsfähigkeit der Blutplättchen auf silikonisierten Objektträgern beobachtet. Diesem Verfahren liegen elektronenmikroskopische Studien BRAUNSTEINER's (5) über das Ausbreitungsverhalten der Thrombozyten zugrunde. Dabei lassen sich zwei Thrombozytenformen differenzieren: einerseits Spinnenformen, auch Reiz- oder Sternformen genannt, bedingt durch die Ausbildung von Pseudopodien, und andererseits ausgebreitete Ruheformen. Die Zuordnung dieser Formen zu quantitativ auswertbaren Größen, welche die Adhäsion oder Aggregation ausdrücken, ist allerdings noch nicht gesichert.

3. Durch Messung des Trübungsverhaltens thrombozytenreichen Plasmas kann man exaktere Einblicke in die Aggregationsneigung der Thrombozyten erhalten (4). Die Zugabe von Adenosindiphosphat (ADP) steigert die Aggregation, worauf die Methode nach VARGA *et al.* (27) beruht. Hier stellt also der Trübungsverlust in der Zeiteinheit das Maß für die Aggregationsneigung der Plättchen dar. Nachdem sich auch *in vivo* ADP, das aus den Thrombozyten freigesetzt wird, an ihrer Aggregation beteiligt, stellt diese Methode ein biologisches Verfahren dar.

Methodik

Die Untersuchungen wurden bei 12 gesunden Normalpersonen und bei 12 Patienten, die wegen einer essentiellen Hyperlipämie in Behandlung der Klinik standen, durchgeführt. Das Kriterium zur Einordnung in die Patientengruppe war der erhöhte Triglyceridspiegel mit Werten zwischen 352 und 3875 mg%. Die Normwerte lagen zwischen 70 und 164 mg%.

Photometrische Trübmessung nach ADP-Zugabe zum Plasma (27). Die Blutabnahme erfolgt morgens vom nüchternen Spender. Das zu untersuchende Blut wurde mittels einer VZA-Kanüle aus der Armvene entnommen und ohne Spritze direkt in einem silikonisierten Glasbehälter aufgefangen. Als Antikoagulans wurde 3,64% Natrium citricum tribasicum benutzt, das dem Blut im Verhältnis 1:9 Teilen vorgelegt wurde. Das Blut wurde bei 1000 U/min 5 min zentrifugiert und das überstehende thrombozytenreiche Plasma in silikonisierte Glasröhrchen abgefüllt. Durch weiteres Zentrifugieren bei 4000 U/min über 15 min wurde thrombozytenarmes Plasma erhalten. Die Bestimmung wurde am Beckmann-Photometer bei einer Wellenlänge von 610 Å durchgeführt. Der Reihenfolge nach wurden Plasma, Veronalpuffer und ADP in die Cuvetten

Tabelle I

Mengenverteilung (in ml) des Plasmas, des Veronalpuffers und der ADP-Lösung in 6 Cuvetten des Photometers.

Cuvette Nr	TAP (ml)	TRP (ml)	Veronal-puffer	ADP (Konzentration in µg/ml) 200	20	2	0,2
1	0,8	–	0,40	–		–	–
2	–	0,8	0,40		–	–	–
3	–	0,8	0,15	0,25	–	–	–
4	–	0,8	0,15		0,25	–	–
5		0,8	0,15	–	–	0,25	–
6		0,8	0,15	–	–	–	0,25

TAP Thrombozytenarmes Plasma, *TRP* Thrombozytenreiches Plasma

des Photometers gefüllt. Die Mengenverteilung ergibt sich aus Tab. I. Unmittelbar vor ihrer Verwendung wurden 4 ADP-Konzentrationen (200, 20, 2 und 0,2 µg/ml Veronalpuffer) hergestellt. Der Inhalt der Cuvetten wurde durch 5-maliges Umkippen gemischt und die Messungen sofort und nach 5, 10, 15, 30 und 60 min durchgeführt. Als Meßwert diente die Licht-Transmission des Plasmas, wobei thrombozytenarmes Plasma als 100 % Wert eingesetzt und das thrombozytenreiche Plasma mit ADP-Zusatz in Prozenten darauf bezogen wurde (Tab. II). Die Thrombozytenaggregation findet ihren Ausdruck in der zunehmenden Klärung des Plasmas nach ADP-Zusatz, d. h. der prozentualen *Zunahme der Licht-Transmission innerhalb von 60 min*. Der erhaltene Wert wurde daher von uns als Delta 60 definiert.

Ausbreitungsfähigkeit der Thrombozyten auf silikonisierten Objektträgern. Zur Ergänzung der angeführten Methode wurde die Ausbreitungsfähigkeit der Thrombozyten und ihre Aggregationsneigung auf silikonisierten Objektträgern nach einem von Bernoff (6) angegebenen Verfahren geprüft. 1,5 ml thrombozytenreiches Citratplasma werden in einem 20-ml-Glasröhrchen, welches in ein Wasserbad mit konstanter Temperatur (37 °C) eintaucht, 10 min lang bei 20 U/min rotiert. Mit dem Plasma wird ein silikonisierter Objektträger 30 min lang überschichtet. Der Objektträger wird anschließend 20 mal in einer Citrat-Kochsalzmischung (1 : 10) geschwenkt, wobei die meisten Erythrozyten und Leukozyten von dem Präparat abfallen. Nach Fixation in 40 % Formol (5 min) werden die Objektträger zur Oxydation 5 min lang in 1/10 Kaliumpermanganatlösung getaucht und nach Abspülen mit Aqua dest. mit einer 1:5 verdünnten filtrierten Giemsalösung gefärbt (60 min). Parallel zur Verarbeitung des rotierten Plasmas wird ein Objektträger mit 1,5 ml nicht rotiertem Plasma überschichtet und in gleicher Weise fixiert und gefärbt. Die Präparate werden mit Hilfe eines Zählnetzes nach Zahl der Thrombozyten, deren Ausbreitungsfähigkeit und dem Vorhandensein von Aggregaten differenziert.

Die Bestimmung der Fettwerte erfolgt aus Vollblut, das je 10 ml durch einen Tropfen Heparin ungerinnbar gemacht und sofort nach Entnahme mit 3000 rpm bei 4 °C 10 min lang zentrifugiert worden war. Das Plasma wurde nach der Methode von Folch *et al.* (12) in der Modifikation von Carlson (10) extrahiert. Im Extrakt erfolgte die Bestimmung des Lipidphosphors nach Bartlett (3) (Lipidphosphor × 25 = Phospholipide), des Gesamtcholesterins nach Searcy *et al.* (24) und der Triglyceride nach Carlson (10). Die Bestimmung der freien Fettsäuren erfolgte nach Dole *et al.* (11) in der Modifikation von Trout *et al.* (26). Die statistische Auswertung erfolgt mit dem Student-Test (16).

Tabelle II

Photometrische Meßwerte (in Prozenten) der Lichttransmission thrombozytenreichen Plasmas n
Zugabe bezogen auf thrombozytenarmes Plasma (= 100%)

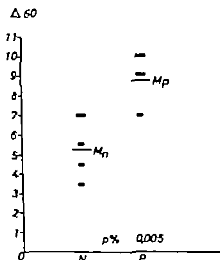
Cuvette Nr	Cuvetteninhalt	Reaktionszeit (min)				
		0	5	10	15	30
1	TAP + Ver Puffer	100	100	100	100	100
2	TRP + Ver Puffer	13,5	13	12,5	12	12
3	TRP + Ver Puffer + ADP 50 µg	18	19	20	21	23
4	TRP − Ver Puffer + ADP 5 µg	17	18	19	19,5	20
5	TRP + Ver Puffer + ADP 0,5 µg	16	16	15,5	15	15
6	TRP + Ver Puffer + ADP 0,05 µg	15	14,5	14	13,5	13,5

Ergebnisse

Die Bestimmung der Thrombozytenaggregation an Hand photometrischer Messungen ergab eine deutliche Differenz der Werte zwischen Normalpersonen und Patienten mit essentieller Hyperlipämie. Wie aus Abb. 1 zu ersehen ist, besteht eine signifikant gesteigerte Aggregation der Plättchen bei Hyperlipämikern gegenüber gesunden Vergleichspersonen ($p = < 0{,}005$). Beide Mittelwerte setzen sich deutlich gegeneinander ab. Bei den Normalpersonen ergab sich eine 60-Minuten-Differenz der Transmission (Delta 60) von 3 bis 7 während letztere bei den Hyperlipämikern 7 bis 10,5 betrug. Die Werte wurden bei einer ADP Konzentration von 200 µg ml erhalten, da sich hier die Thrombozytenaggregation am ausgeprägtesten zeigt. Tab. III gibt eine Übersicht über die Korrelation zwischen der Fettfraktion des Plasmas und der Thrombozytenaggregation bei 12 Patienten mit essentieller Hyperlipämie und 12 Normalpersonen.

Auch die Auswertung der Thrombozytenpräparate nach Überschichtung silikonisierter Objektträger mit thrombozytenreichem Plasma zeigte eine signifikante Differenz beider Untersuchungsgruppen.

Bei den Hyperlipämikern fanden sich in 50% der Fälle zahlreiche Aggregate, die im rotierten Plasma besonders deutlich zum Vorschein kamen (Abb. 2). Neben dem Nachweis der Aggregate wurden je 1000 Zellen in ausgebreitete Ruheformen und Spinnen- oder Reizformen differenziert. Dabei fanden wir, daß bei den Normalpersonen im Mittel 56 der Plättchen ausgebreitet waren, gegenüber den Hyperlipämikern mit im Mittel nur 29° ausge-



Δ60 = Differenz der Transmission % von thrombozytenreichem Plasma nach Zusatz von ADP sofort und nach 60 min
M_n = Mittelwert Normalperson
M_p = Mittelwert Hyperlipämiker
N = Normalpersonen
P = Hyperlipämiker
p% = Signifikanzniveau

Abb. 1 Thrombozytenaggregation unter ADP (200 μg/ml) bei Normalpersonen und Hyperlipämikern

breiteten Formen (Abb 2 und 3) (Die Werte beziehen sich auf rotiertes thrombozytenreiches Plasma.)

Diskussion

Aus den vorliegenden Untersuchungsbefunden können die folgenden Schlüsse gezogen werden

1 Der Zusammenhang zwischen erhöhten Triglyceridwerten bei essentieller Hyperlipämie und einer vermehrten Aggregationsneigung der Thrombozyten unter ADP bestimmt durch Trübungsmessungen nach Vainer *et al* (27) ist signifikant gesichert. An Hand einer weiteren Patientengruppe mit nur mäßig erhöhten Triglyceridwerten werden wir abzuklären trachten, ob eine direkte Korrelation zwischen den beiden Werten besteht.

2. Die Untersuchungen nach der Methode von Breddin (8) ergab ebenfalls eine signifikante Beziehung zwischen vermehrter Aggregation der Plättchen und Hyperlipämie. Es fanden sich einer

seits Thrombozytenaggregate, anderseits auch vermehrte «Sternformen». Ob diese «Sternformen» mit einer erhöhten Adhäsion in Zusammenhang stehen, ist noch zu untersuchen. Weitere Untersuchungen mit der Methode nach SALZMAN (22) sind in Vorbereitung.

3. Die Frage, durch welchen Mechanismus ein hyperlipämisches Plasma zu einer Steigerung der Thrombozytenaggregation führt, läßt sich aus den vorliegenden Untersuchungen noch nicht beantworten.

Neben den Triglyzeriden könnten auch andere, bei essentieller Hyperlipämie erhöht gefundene Fettfraktionen einen Einfluß auf die Thrombozytenaggregation ausüben. Nach MUSTARD *et al.* (19)

Tabelle III

Gegenüberstellung von Fettwerten des Plasmas (Cholesterin, Triglyceride, freie Fettsäuren, Phospho[illegible] und Thrombozytenaggregation.

(Delta 60 Differenz der Transmission sofort und nach 60 min).

Lfd. N	Name	Chol.	T G	F F S.	Ph. L.	Delta 60	% ungebreit. Tz. Formen	Aggregate nach Rot.	T [illegible]
1	R. R.	145	52	840	219	5,5	51	—	[illegible]
2	S. K.	228	118	615	271	5,5	74	—	[illegible]
3	R. B.	123	80	750	194	4,5	57	—	[illegible]
4	F. L.	197	164	535	202	7,0	53	—	[illegible]
5	J. S.	156	60	420	207	7,0	51	—	[illegible]
6	L. Z.	174	27	595	190	6,5	59	—	[illegible]
7	G. H.	250	56	475	281	6,0	57	—	[illegible]
8	J. H.	320	65	540	294	7,0	53	—	[illegible]
9	J. S.	182	152	492	317	3,5	57	—	[illegible]
10	P. M.	207	45	570	160	4,5	59	—	[illegible]
11	L. Z.	200	38	1050	240	3,5	63	—	[illegible]
12	L. S.	230	72	630	247	4,0	57	—	[illegible]
1	K. S.	485	1725	775	530	10,0	23	—	[illegible]
2	F. L.	540	1630	830	630	7,5	21	+	[illegible]
3	F. L.	320	410	920	350	7,5	48	+	[illegible]
4	J. E.	362	1175	890	472	10,5	18	+	[illegible]
5	N. R.	368	2170	1140	412	7,0	28	—	[illegible]
6	H. B.	563	610	900	530	8,0	32	—	[illegible]
7	A. S.	220	410	690	270	9,0	32	+	[illegible]
8	G. S.	212	35	2860	300	7,0	44	—	[illegible]
9	W. K.	235	620	970	386	9,0	31	++	[illegible]
10	T. M.	276	625	1440	335	10,0	23	++	[illegible]
11	L. A.	615	1120	820	640	9,0	—	—	[illegible]
12	G. K.	960	3875	1240	835	9,5	15	—	[illegible]

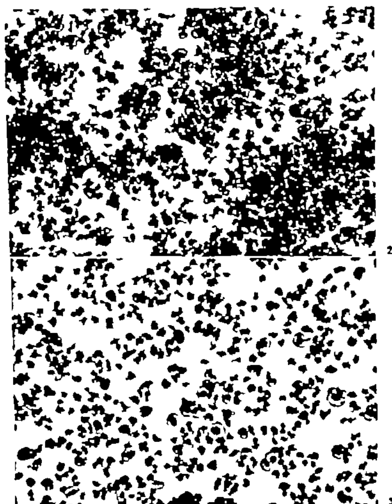


Abb. 2. Thrombozytenaggregate bei Patienten mit Hyperlipämie (nach Rotation des Plasmas) «Spinnenformen» vermehrt.
Abb. 3. Vorwiegend ausgebreitete Thrombozytenformen bei Normalpersonen (nach Rotation des Plasmas)

sowie HASLAM (13) besteht eine Korrelation zwischen erhöhten freien Fettsäuren und der Aggregationsneigung der Thrombozyten. Es bleibt allerdings abzuklären, ob bei diesen Untersuchungen nicht auch eine Erhöhung der Triglyzeride bestanden hat. KERR *et al.* (15) beobachteten unter der Einwirkung von gesättigten Fettsäuren eine Plättchenaggregation, die um so stärker war, je mehr C-Atome die Kette bildeten. Die Aggregation war schließlich

irreversibel und durch ADP nicht mehr beeinflußbar. Ungesättigte Fettsäuren führen zu einer raschen, jedoch reversiblen Aggregation. Bei den Untersuchungen von KERR *et al* (15) wurde weiterhin bereits durch geringe Mengen von Phospholipiden, mit Ausnahme von reinem Lezithin, eine deutliche Steigerung der Aggregation der Thrombozyten gesehen. Zur Abgrenzung der Wirkung der verschiedenen Fettfraktionen sind Untersuchungen im Gange.

Schließlich ist es jedoch auch vorstellbar, daß die Hyperlipämie durch einen indirekten Mechanismus, beispielsweise durch die Erhöhung des Fibrinogenspiegels, zur erhöhten Thrombozytenaggregation führt.

Zusammenfassung

Es wird über vergleichende Untersuchungen der Thrombozytenaggregation bei 12 Patienten mit essentieller Hyperlipämie und 12 Normalpersonen berichtet. Die Hyperlipämiker wiesen eine signifikant gesteigerte Aggregation der Thrombozyten auf. Morphologisch fanden sich neben Aggregaten vermehrt Spinnen- oder Sternformen.

Summary

Comparison of platelet aggregation in 12 patients with essential hyperlipaemia and 12 normal subjects is reported. Hyperlipaemic patients present significantly increased platelet aggregation. Morphologically numerous spider or star formations were seen in addition to simple aggregates.

Résumé

L agrégation des lymphocytes chez 1 malades tteints d'hyperlipémie essentielle été comparée à celles d 12 personnes normales. L agrégation des thrombocytes était nettement ugmentée chez les hyperlipémiques. L examen morphologique montra à part les aggrégats un nombre plus grand des thrombocytes en forme d araignée et d'étoile

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Experimental Studies on the Mechanism of Action of Benzene on the Bone Marrow (Radioautographic Studies Using ^{3}H Thymidine)*

S Moeschlin and B Speck

Despite the fact that the use of benzene as a solvent is prohibited in most countries there are still many reports on chronic benzene intoxications (1 2 3 4 5) The most frequently quoted sign is aplastic anemia but also there has been a high incidence of leukemia which generally has been of the acute myeloblastic type. The elective toxic effects of benzene on the bone marrow and the persisting inhibition of the proliferation of the marrow cells even after stopping benzene exposure still remain unanswered questions (6a)

In the present study the mechanism of action of this substance on the bone marrow cells is examined with tritiated thymidine.

Methods

Sixty rabbits with body weight of 1 1 to 3 7 kg and of both sexes were examined. I preliminary studies 28 animals received benzene in oily solutions subcutaneously This group of animals could not be evaluated statistically since there was wide variation of resorption and of the resulting toxic effects. Another group of 5 animals was given single injection of 2 0 ml/kg of pure benzene subcutaneously

After the initial technical difficulties had been solved, main experiment was started with 27 animals This group was intoxicated with subcutaneous injections of pure benzene three times weekly It should be mentioned at this point, that the difference between the lethal dose and the one inducing pancytopenia was small. I our animals, the optimal dose of pure benzene proved to be in th order of 0.3 ml/kg/day) With this dose severe pancytopenia could be induced in all the nimals within one to nine weeks, except for those dying from early anaphylactic or infectious complications. As soon as the leukocyte count in the peripheral blood dropped below 3000/mm^3 0 5 μc of ^{3}H-methyl-thymidine (New England Nuclear Boston, Mass.) were given intravenously The specific activity of this pyrimidine nucleoside labelled w th tritium was

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6.7 C/mM throughout. After an incubation period of exactly 60 min, the animals were anestetized. Bone marrow was aspirated from the epiphysis of the femur. Thin slides were then prepared from solid marrow particles. After air drying, they were fixed in absolut ethanol for 10 min and then covered with Kodak AR 10 stripping film in the dark-room. The preparations were then exposed for 20 days at 4 °C. Subsequently they were developped with Kodak D-19 developper and Kodak fixator at 18 °C. The staining was performed through the film with Giemsa solution buffered to pH of 6.5. A total number of 19 animals was studied radioautographically. In all these animals 500 baso-philic and 500 polychromatophilic normoblasts were enumerated and the percentage of labelled cells was calculated. The pronormoblasts [= K_1 stage according to Wickramasinghe (7)] and the macronormoblasts (= K_2) could be definitely quantitated only in three intoxicated animals, and the myelocytes only in 2 animals. Cells were considered to be labelled if the number of grains over their nucleus was more than three times the background graincount over an equal area.

Results

Peripheral blood. With a dose of 0.3 ml/kg/day of pure benzene the time span until a peripheral pancytopenia was induced varied from 1 to 9 weeks. A single injection of 2.0 ml/kg did not cause any remarkable changes of the peripheral blood counts during a 6 weeks control period.

The differential counts of the leukocytes showed a slight drop of the lymphocytes and a slight increase of basophils and eosino-phils. If the relative percents of the different cell lines were cal

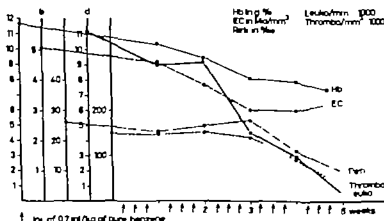


Fig. 1. Representative animal from the main experiment. 0.3 ml/kg/day of pure benzene was given subcutaneously in three weekly injections. Note the drop of the peripheral leukocyte, thrombocyte and reticulocyte counts; they generally occurred in the second to the fourth week. There were considerable differences of sensitivity, though. The drop of the erythrocyte count and of the hemoglobin is relatively small.

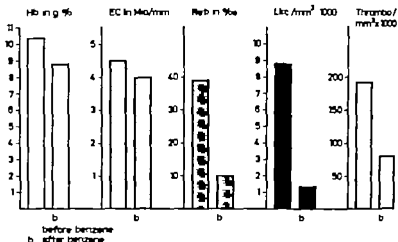


Fig. 2. Mean peripheral blood counts of 19 animals of the main experiment which came to radioautography. The bars at the left represent the values before benzene was injected, the ones on the right the values at the time radioautography was performed. Note again *the striking drop of the leukocyte, thrombocyte and the reticulocyte counts* with an only slight drop of the erythrocytes and the hemoglobin levels.

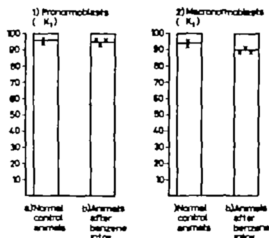


Fig. 3. Labelling of the young erythroid precursors. Note that there is only drop of 95.5 to 94.5 labelling at the pronormoblast (= K_1) stage and of 94 to 90% at the macronormoblast (= K_2) stage in normal animals compared to animals intoxicated with benzene.

culated in absolute numbers of cells per mm³ a decrease of all elements was found which was most striking in the lymphocytes.

Bone marrow. Despite the fact that all the animals had a peripheral pancytopenia after exposure to benzene, there was a considerable difference in the morphologic marrow picture. Of the examined 19 marrows 4 were very hypoplastic, 6 hypoplastic, 5 of average

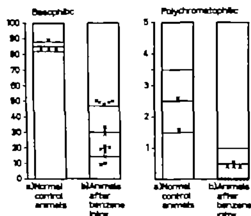


Fig 4 The radioautographic findings of 4 normal animals are opposed to the findings in 19 animals intoxicated with benzene. Note the significant drop of labelling of the *basophilic normoblasts* (= $K_{3_{II}}$) from 84.5 % (s.D 2.6 %) in normal animals to 30.2 % (s.D 16.5%) in the intoxicated animals. This decrease of labelling indicates clearly the diminution of the proliferative potential of the bone marrow cells at this stage of maturation. At the stage of the *polychromatophilic normoblast* (= K_{3_4}) the labelling dropped to 0.52% (s.D 0.49%) in the intoxicated animals as compared to 2.5% (s.D 1.05) labelling in the normal controls.

cellularity and 4 were very cellular There was no correlation between the cellularity and the duration of exposure to benzene The diminution of the *myeloid precursors* was remarkable. The *erythroid line* was generally quantitatively well preserved but there were a number of qualitative aberrations. Numerous pathologic changes of the chromatin structure were found A shift to the left in erythropoesis with the appearance of very immature cells (= erythrogones) with giant nuclei was another characteristic feature. The interpretation of paraffin sections of the marrow of an intoxicated animal by Institute of Pathology of the University of Zurich (Prof UEHLINGER) was in agreement with our findings Predominant reduction of myelopoesis with maintained erythropoesis and appearance of erythrogones

Radioautographic findings The young erythroid precursors (pronormoblasts = K_2) and the macronormoblasts (= K_1) could be quantitated only in a few of the cellular preparations. At this stage of the development there was no significant difference between the amount of labelled cells in the normal animals and in the intoxicated ones. At the stage of the *basophilic normoblast* (= K_{3_2}) though there was a very pronounced drop of labelling in the in-

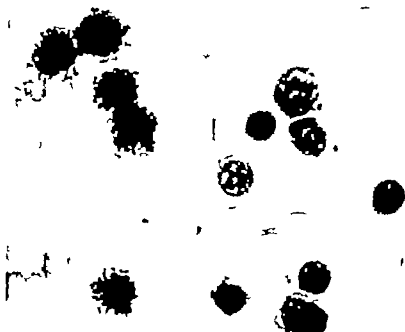


Fig 5 ( ) Radioautographic plate from the marrow of a normal animal. Note the distinct labelling of the basophilic normoblasts.
(b) Radioautographic plate from an intoxicated animal made under exactly the same conditions. Note the lack of labelling of most of the erythroid precursors. Below there is [illegible] myelocyte which is not labelled either. The background grain count is low in both preparations.

toxicated animals, which persisted at the stage of the polychromatophilic normoblast.

The myeloid line was so markedly reduced after exposure to benzene, that only in two animals 500 cells could be quantitated. At stage of the myelocyte the labelling was decreased from 67 % in normal animals to 7 3 (s. D 2 1%) in the intoxicated ones.

Discussion

Our radioautographic studies prove that the *myelotoxicity of benzene and the resulting pancytopenia are caused by a very pronounced inhibition of DNA synthesis*. The ^{3}H thymidine which was used for *in vitro* incubation is a direct precursor of DNA. During an incubation period of 60 min all the cells which are in the process of active DNA-synthesis are labelled (8–10). Therefore *the amount of labelling of the different cell types is a direct index for their proliferative potential*. In the *erythroid line* the proliferation of the pronormoblasts

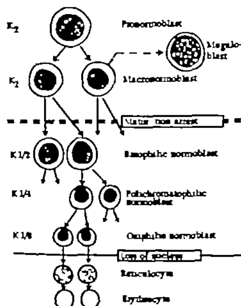


Fig. 6. Model of erythropoesis (WICKRAMA). The maturation arrest in erythropoiesis during chronic benzene intoxication must be between the stages K_2 and $K_{1/2}$

(= K_2) and of the macronormoblasts (= K_2) was in the normal range as far as this could be quantitated. On the other hand there was a very pronounced *lack of proliferative potential at the stage of the basophilic normoblast* (= $K_{1/2}$) *in all the animals*. This finding indicates a *maturation arrest* between the macronormoblast (= K_2) and the basophilic normoblast (= $K_{1/2}$). Similar observations have been made in other intoxications, e. g. lead (6b).

In the *myeloid line* also there was a marked decrease of the proliferative potential during benzene intoxication. It was quantitated at the stage of the myelocyte. The short survival time of the leukocytes and of the thrombocytes in the peripheral blood and the rapid maturation of the reticulocytes explain the early drop of these blood counts following severe inhibition of DNA-synthesis. On the other hand the erythrocyte counts and the hemoglobin levels remained relatively constant during benzene intoxication. This is due to the longer survival of the red cells in the peripheral blood. The fact that the human bone marrow may become aplastic as well as hyperplastic during chronic benzene poisoning is well known (6a, 11).

The appearance of erythrogones as we found them in the intoxicated animals, has previously been observed during treatment with antimetabolites (12 13) aplastic crises of hemolytic anemias and in pernicious anemia (14)

Addendum. Since this work has been completed, additional radioautographic studies have been performed in our laboratory using ³H-cytidine and ³H-uridine. Our preliminary data indicate that in addition to the inhibition of DNA-synthesis, there may be an inhibition of RNA-synthesis of the bone marrow cells during chronic benzene intoxication.

Summary

In rabbits intoxicated by means of subcutaneous benzene injections, autoradiographic studies of the labelled bone marrow cells after intravenous incubation with ³H-thymidine were performed. There was a marked individual difference of sensitivity to the same dose of benzene. A severe peripheral pancytopenia resulted in all the animals within one to nine weeks. The bone marrow became hypoplastic in about one half of the animals and it remained cellular in the other half. This is in agreement with the findings in chronic human benzene intoxication. For the first time it was shown radioautographically with ³H-thymidine that the pancytopenia of chronic benzene poisoning is due to a severe inhibition of DNA-synthesis in the bone marrow cells. The severe disturbance of DNA-synthesis may also be one of the factors which could give rise to the development of leukemia.

Zusammenfassung

Bei Kaninchen mit experimenteller Benzolvergiftung wurde das Knochenmark autoradiographisch untersucht. Die individuelle Empfindlichkeit auf eine gleiche Benzoldosis schwankt erheblich. Bei dreimaligen s.c. Benzolinjektionen pro Woche kam es nach 1 bis 9 Wochen zu einer schweren Panzytopenie. Das Knochenmark wurde bei der Hälfte der Tiere hypoplastisch, bei den übrigen war es noch zellreich. Dieses Ergebnis stimmt mit den Beobachtungen bei der menschlichen Benzolvergiftung überein. Erstmals konnte durch autoradiographische Untersuchung mit ³H-Thymidin eine schwere Hemmung der DNS-Synthese als Ursache für die gehemmte Blutzellbildung bei der Benzolvergiftung nachgewiesen werden. Die schwerwiegende Störung der DNS-Synthese konnte ein Faktor sein, der die leukämische Entartung des Knochenmarks bei der chronischen Benzolvergiftung begünstigt.

Résumé

La moelle osseuse de lapins intoxiqués par des injections sous-cutanées de benzol a été étudiée par autoradiographie après l'injection intraveineuse de thymidine tritiée. La sensibilité individuelle à la même dose de benzol varie considérablement. Une pancytopénie périphérique sévère apparut chez tous les animaux au bout de une à neuf semaines, les injections étant administrées à raison de trois par semaine. La moelle osseuse devint hypoplasique chez à peu près la moitié des animaux; chez les autres, elle demeura riche en cellules. Ces résultats concordent bien avec les constatations faites dans les cas d'intoxication au benzol chez l'homme. Pour la première fois, il a été démontré par autoradiographie à l'aide de thymidine tritiée que la pancytopénie de l'intoxication chronique au benzol est due à une inhibition sévère de la synthèse de l'ADN. Cette inhibition est peut-être aussi l'un des facteurs pouvant favoriser l'apparition d'une leucémie lors d'une intoxication chronique au benzol.

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Histochemical Enzyme Analysis of Peripheral Blood Changes in Murine Virus-Induced Leukemia*

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The occurrence of an abnormal cell, designated as a V-cell, was reported recently by Hopkins and Siegel (7) in the peripheral blood of mice infected with Rauscher murine leukemogenic virus. The authors suggested a possible lymphocytic origin for the V-cell, in that its presence in differential smears was usually accompanied by numerous atypical lymphocytes and smudge cells. This unusual cell has thus far been reported in one other virus-induced blood dyscrasia of mice, that of Friend virus-infected animals (8).

In a more recent report Weaver *et al.* (17) noted that lactic dehydrogenase (LDH) activities in the sera of Rauscher virus-infected mice were 20 to 25 times that of control animals. This increase in LDH was coterminous with augmented total nucleated cell counts and increased spleen and liver weights. Along these lines, increased alkaline phosphatase has been noted histochemically in hepatic sinusoidal epithelium in Friend leukemic animals (16) and histochemical analysis of the spleens (9) and thymuses (15) of AKR strain mice have demonstrated increased lymphocyte alkaline phosphatase in mice developing spontaneous lymphocytic lekemia.

In the present histochemical investigation, lactic dehydrogenase, acid and alkaline phosphatase and concurrent cytological studies of blood smears from normal and Rauscher virus-infected mice were carried out chronologically with leukemic development.

* This study was supported in part by USPHS Grant No. CA 10089 from the National Cancer Institute.

Materials and Methods

The virus (13) employed in this study had been through seven serial mouse passages in this laboratory since its receipt from Dr FRANK J RAUSCHER of the National Cancer Institute Extracts of virus-induced leukemic spleens were twice clarified by centrifugation, each time at 2500 rpm for 20 min in an International PR 2 refrigerated centrifuge Two-tenths ml of 10 percent suspension in Hanks balanced salt solution containing 100 units of penicillin and 100 μg of streptomycin per ml was inoculated into eight 4 to 5 week old female BALB/ mice obtained from Jackson Memorial Laboratory Virus-inoculated and control mice were bled weekly from the retro-orbital plexus. Blood picture changes, including nucleated cell counts, differentials and histochemical analyses were followed and records kept for each individual animal. Dehydrogenase histochemical analysis of cells in unfixed blood films was observed to be hampered by the tendency of cells to float off when immersed in the substrate. Fixation with acetone (12) preserved the cells better for photography whereas trapping the cells under coverslip and sealing with vaseline (1) gave better dehydrogenase reaction. The procedure described below was found to preserve the cells well and to give good histochemical reaction, although counterstaining and photographic representation proved difficult. Blood films were prepared for the NAD-linked lactic dehydrogenase histochemical analyses by first forming raised ring of Permount ® on the slide containing the fresh air dried blood smear Substrate solution was then added within the area of this ring and coverslip applied to effectively establish permanent seal. The substrate concentrations employed were essentially those of PEARSE (11) Acid phosphatase analyses was carried out on blood smears by modified NOVIKOFF procedure (10), employing alpha-beta-glycerol phosphate and 0.2 M sucrose as an osmotic agent. Alkaline phosphatase analysis was done using the naphthol AS-MX phosphate and red-violet LB salt as described by BURSTONE (5)

Results and Discussion

In Table I is recorded the percentage of lymphocytes showing different degrees of LDH activity for individually infected animals over a period of 5 weeks following Rauscher virus inoculation The range of values for 4 normal animals was included for the purpose of comparison At week one the peripheral smears of the inoculated animals, with the exception of animal R2 and animal R8, were identical with those of the control group Animals R2 and R8 demonstrated increased numbers of lymphocytes with an enlarged eccentric cytoplasmic rim. These large lymphocytes showed a more intense LDH activity than normal, characterized by increased clumping of formazan in the eccentric cytoplasmic areas (Fig 3) As the disease developed, lymphocytes in Rauscher virus-infected animals showed, with the exception of animal R2, an augmented lactic dehydrogenase activity Fig 1 shows this diagrammatically Animal R2 by the 4th week returned to a normal blood picture and had a normal lactic dehydrogenase response the blood picture remained normal through the 9th week, but the animal expired at

Table I

Lymphocyte LDH activities in normal and Rauscher virus-infected mice during the first five weeks following virus inoculation.

Animal number	LDH intensivity[1]	Percent lymphocytes showing given degree of LDH activity week I	week II	week III	week IV	week V
R 1	+	2	2	1		
	++	93	91	39		
	+++	5	7	50		
	++++	0	0	10[a]		
R 2	+	1	2	2	1	2
	++	91	73	96	96	97
	+++	8	25	2	3	1
	++++	0	0	0	0	0
R 3	+	1	2	1	0	0
	++	97	92	69	64	43
	+++	2	5	30	35	50
	++++	0	0	0	1	7[a]
R 4	+	2	2	0	0	0
	++	95	93	89	68	63
	+++	3	5	20	32	37
	++++	0	0	0	0	0
R 5	+	2	1	0		
	++	96	84	25		
	+++	2	15	62		
	++++	0	0	13[a]		
R 6	+	3	4	1	0	0
	++	95	90	74	33	15
	+++	2	6	25	57	71
	++++	0	0	0	16[a]	14[a]
R 7	+	2	0	2		
	++	94	97	61		
	+++	4	3	28		
	++++	0	0	9[a]		
R 8	+	0	2	0		
	++	89	66	11		
	+++	11	32	76		
	++++	0	0	13[a]		
Range of controls	+	1—3	1—4	0—5	0—4	2—4
	++	94—97	95—98	95—98	91—89	95—97
	+++	1—4	0—3	0—3	0—4	1—3
	++++	0	0	0	0	0

Symbols used, + to ++++ denote varying degrees of reaction from minimal to maximum.

X-cells identified in Wright-Giemsa preparations.

Animal died between weeks III and IV.

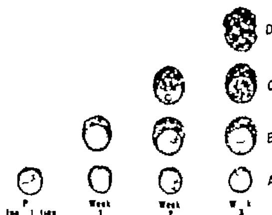


Fig. 1 Diagrammatic representation of the time sequence of lymphocyte LDH augmentation observed in the majority of the peripheral blood smears of Rauscher virus-infected animals.

(A) Normal appearing lymphocyte showing ++ LDH staining.
(B) Lymphocyte with increased cytoplasm and ++ LDH staining.
(C) Lymphocyte with increased +++ LDH staining.
(D) Cell showing maximal ++++ LDH activity which appeared in the same animals having V-cells identified by Wright-Giemsa staining.

All figures on this plate show NAD-linked lactic dehydrogenase (LDH) activity employing Nitro-BT as hydrogen acceptor X 2000. Preparations were unfixed and not counterstained.

the 16th week with a typical erythroleukemia. Animal R6 continued with a high level of LDH activity through the 9th week, expiring at the 16th week with a total nucleated cell count of 800000 and a blood picture resembling the lymphocytic response described by RAUSCHER (13).

Wright-Giemsa stains of the blood films, 3 weeks post-inoculation showed the presence of V-cells in animals R1, R5, R7 and R8. The V-cell, described in detail by HOPKINS and SIEGEL (7) is a densely basophilic staining cell of 7—20 nm in diameter with an indistinct nuclear membrane. The V-cell in the LDH preparations contained large irregular masses of formazan precipitate obliterating much of the morphological detail (Fig. 6, 7).

A slight increase in the number of lymphocytes with cytoplasmic protrusions (budding) was observed during the first week of infection. By the 2nd week following virus inoculation, a great increase in the percentage of lymphocytes exhibiting cytoplasmic budding was noted in most of the infected animals. At 3 weeks, 7 of the 8 animals exhibited blebbing or cytoplasmic budding on

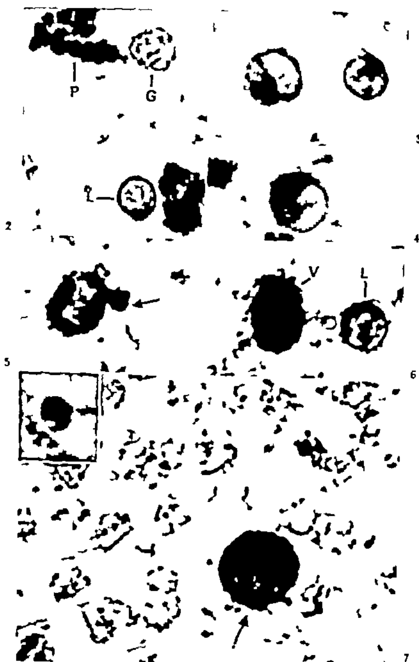


Fig. 2. A lymphocyte (L), granulocyte (G) and platelet clumps (P) from peripheral blood of normal BALB mouse. Typical LDH activity is observed in the lymphocyte as diffuse moderate cytoplasmic staining and as dense area. The granulocyte appears essentially unreactive.

25 to 40 % of the lymphocytes, while in the normal animals this phenomenon rarely exceeded 0.5 %.

The virus-infected mice, except for R2 developed an erythroleukemia as described by SIEGEL (14) Reticulocytosis, anemia, and an increase in nucleated cell counts reached a maximum at 4—6 weeks. The normal hematological data and the values observed in Rauscher virus-infected animals were comparable to several hundred similarly studied animals observed in this laboratory over a period of several years* Based on these studies, it was found that 4 8 % of the virusinoculated animals were refractile to the virus, 0 7 % had a delayed erythrocytic response, and 3.5 developed a late lymphocytic pattern similar to that described by RAUSCHER (13 14) The erythrocytic response was seen in 85.5 of the animals. The presence of LDH activity in erythrocytes was manifested by dye granules on the cell membrane (Fig 7) BOHX (4) has shown that this is a true erythrocytic enzyme response and not due to LDH presenet in the serum. Reticulocytes which are present in small numbers in normal animals often reached 50 % of the erythrocytic population in the virus-infected mice, and showed a more intense LDH activity by manifesting what appeared to be intra cellular as well as surface dye deposits. Metarubricytes appeared in the peripheral blood at 3—4 weeks in the leukemic animals (Fig 7) LDH activity of the pyknotic nucleus of these cells showed intense staining, while the cell membrane remained relatively unreactive.

Acid phospatase analysis was conducted the 4th and 5th weeks. The majority of the lymphocytes from RAUSCHER infected

Fig 3. Two lymphocytes seen in blood of Rauscher virus-infected animal. Some elevation of LDH activity is observed in small lymphocyte while the larger one shows an increased cytoplasmic nuclear ratio, eccentric nucleus and more dense formazan clumping.

Fig 4. A large lymphocyte, seen later in the disease process, showing intense LDH activity in the enlarged cytoplasmic area.

Fig 5. A large atypical lymphocyte showing large bleb (→) LDH activity is noted to be marked.

Fig 6. V-cell with intense LDH activity (V) and small lymphocyte (L)

Fig 7. A V-cell showing intense LDH activity with smaller deeply staining blebs (→ extending from the cell. Reticulocytes are numerous in the background with their evident LDH staining. A metarubricyte with intense staining of the central area is shown in the insert.

* Unpublished data

animals showed augmented acid phosphatase activites compared to those from control animals. Ninety % of the lymphocytes from normal animals were negative in enzyme activity and 10 showed one granule of acid phospatase staining. All of the surviving Rauscher infected animals at weeks 4 and 5 except R2 had 20—40 of the lymphocytes showing moderated to intense acid phospatase activity. Though METCALF (9) and SMITH (15) noted increased lymphocyte alkaline phosphatase activity in AKR mice developing spontaneous lymphocytic leukemia, no such increase could be demonstrated in the BALB/c mice in response to the Rauscher virus erythroleukemia. Tests for akaline phospatase reactions were conducted weekly for a period of 5 weeks. The presense of this enzyme was not demonstrable in the peripheral blood cells of either the Rauscher infected animals or of the control animals. For further verification normal rat peripheral blood smears were incubated in the same substrate. These demonstrated intense alkaline phospatase activity in most of the granulocytic cells, confirming the validity of the negative test.

In general with development of the erythroleukemic state the increase in percentage of lymphocytes with augmented LDH activity was usually concurrent with the appearance of lymphocyte blebbing and V-cells. Blebbing under these conditions has been thought to indicate impending cell death as sugested by BESSIS (2). The increased cellular LDH activity in the lymphocytes and V-cells and the increased numbers of immature cells of the erythrocytic series with their high levels of LDH may both contribute to the previously noted elevation of serum LDH (17). It would appear from the present findings that the occurrence of V-cells along with increased lymphocyte budding in the early weeks of Rauscher virus infection may be indicative of impending death of the animals. Such a phenomenon might prove useful in laboratory sampling of those animals with low prospects of survival.

Summary

Cytological studies and histochemical enzyme analyses were carried out chronologically on individual mice following inoculation with Rauscher leukemogenic virus. A marked increase in cytoplasmic blebbing of lymphocytes was noted during the development of the disease along with the appearance of V-cells, and immature cells of the erythrocytic series as the disease progressed. The majority of lymphocytes from infected animals showed an increased acid phosphatase reaction, while alkaline phosphatase activity remained negative as in the normal controls.

Zusammenfassung

Bei Mäusen wurden nach Inokulation mit dem leukämogenen Rauscher Virus chronologisch zytologische Untersuchungen und histochemische Enzymanalysen vorgenommen. Während der Entwicklung der Krankheit fand sich eine deutliche Zunahme der Zytoplasmaausstülpungen von Lymphozyten zugleich mit dem Auftreten von V-Zellen. Mit dem Fortschreiten der Erkrankung ergab sich ein starker Anstieg der LDH-Aktivität in Lymphozyten, V-Zellen und unreifen Zellen der erythrozytären Reihe. Die Mehrzahl der Lymphozyten infizierter Tiere zeigte einen Anstieg der sauren Phosphatase, während die Aktivität der alkalischen Phosphatase wie bei den normalen Kontrollen negativ blieb.

Résumé

Après l'inoculation du virus leucémogène de Rauscher à des souris, il a été procédé plusieurs fois chez chaque animal à des examens cytologiques et à des analyses histochimiques. Une nette augmentation du nombre des excroissances cytoplasmatiques des lymphocytes a été enregistrée au cours de la maladie en même temps que l'apparition de cellules V ainsi que de cellules immatures de la lignée érythrocytaire. Durant la progression de la maladie, l'activité de la LDH augmenta fortement dans les lymphocytes, les cellules V et les formes immatures de la lignée érythrocytaire. La majorité des lymphocytes d'animaux infectés montra une augmentation de la phosphatase acide, l'activité de la phosphatase alcaline restant nulle comme chez les animaux de contrôle.

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The Types of Rat Thoracic Duct Lymphocytes which Respond to Phytohemagglutinin in vitro

W O RIEKE and M. R. SCHWARZ

While there is ample evidence that phytohemagglutinin (PHA) causes the *in vitro* transformation of small lymphocytes from the human (1 2 3 4 5) and rat (6 7 8) there remain important questions about the type of small lymphocytes which respond These questions arise from the demonstration that small lymphocytes (at least in the rat) are of two types with different circulating life spans (9 10 11) These types have been identified by their labeling patterns in rats injected with H^3 thymidine and include a group of short lived small lymphocytes which circulate for only 1—2 weeks, and another group of long lived, small cells which circulate for periods up to several months (8, 9 10 11 12) Short and long lived small lymphocytes from various lymphoid tissues of the rat have recently been labeled and cultured by METCALF and OSMOND who report that members of both groups enlarge when stimulated with PHA (8) The present study confirms and extends their report by noting that while both long and short lived thoracic duct lymphocytes enlarge when stimulated with PHA, the short lived population is proportionately more responsi e

Materials and Methods

(1) Animals

Male Lewis rats weighing 80 to 100 g were injected with H^3-thymidine so as to label mainly the short-lived or only the long-lived small lymphocytes which then were collected from the thoracic duct in the neck or bdomen.

( ) *Short-lived lymphocytes.* As in previous work (11 13) short-lived small lymphocytes were labeled by administering H^3 thymidine four times day for ery few days. Because this schedule also labels large and medium cells, tw control procedures were followed to correct for ny enlarged forms hich may ha derived in cultures from these pre-existing labeled large and medium lymphocytes. In the first of these the collected

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Cytogenetic Studies in Unusual Forms of Chronic Myeloid Leukaemia

M. W. Elves and M. C. G. Israëls

Since the original discovery of the abnormal small metacentric chromosome, replacing a normal group 21–22 autosome in patients with chronic myeloid leukaemia (17) many confirmatory reports of this association have appeared (2–15). This chromosomal abnormality has been named the Philadelphia Chromosome (Ph[1]) and is now regarded as being diagnostic of chronic myeloid leukaemia. In this paper we present 11 cases of unusual forms of leukaemia or allied diseases in which cytogenetic examination has contributed significantly to correct diagnosis.

Methods

For cytogenetic studies of human leukaemia and allied disorders we have preferred direct bone marrow technique. Peripheral blood has been used on occasions, particularly in the earlier studies (5), but has the drawback of not being truly representative of the marrow cell population. Our technique has been developed in this laboratory over the past 5 years and gives good results in most cases of myeloproliferative disease.

Marrow is aspirated from the sternum, after induction of local anaesthesia, into 20 ml siliconed all-glass syringe containing 15 ml of sterile culture medium 199. About 1–2 ml of marrow is usually obtained without difficulty; aspiration directly into culture medium obviates the need for an anticoagulant in the cultures. The volume of the culture is made up to 20 ml with 199 and 0.6 mg of Colcemid is added. The culture is incubated for 1½–2 h at 37 °C. After this time a fragile clot is often found in the culture vessels but is easily dissolved by vigorous shaking and does not give rise to difficulty. The cell suspension is then centrifuged gently at 800 r. p. m. for 15 min, subjected to hypotonic treatment in 0.2% saline (w/v) for 20 min at 37 °C, recentrifuged, and fixed in a mixture of 4 parts absolute ethyl alcohol and 1 part glacial acetic acid. Fixation was carried out with great care in order to prevent clumping of the cells and the following technique is recommended. The unfixed cells are first completely resuspended in a few drops of culture medium, then about 2 ml of fixative is added drop by drop whilst agitating the cells and then the mixture is kept at 4 °C for 30 min. A new change of fixative is now made and the cells left at low temperature for 12–24 h. For spreading, the fixative is removed and the cells resuspended in a mixture of 4 parts glacial acetic acid and 1 part absolute

ethyl alcohol to give a faintly milky suspension. This suspension is dropped onto clean glass slides previously chilled with solid CO_2, rapidly air-dried over a flame and stained with Giemsa stain (Gurr' R 66) for 10 min. Photography was facilitated using 'Micro-neg pan film (Ilford, 35 mm) and a light green filter Prints were made on hard grade bromide paper

Owing to the low mitotic index found in the marrow of many patients with myeloproliferative disease we now give the patient an intravenous injection of the periwinkle alkaloid vincoleucoblastine (Velbe Lilly) 10 mg is given 2 or 3 h before the marrow aspiration longer intervals give rise to many 'Colchicine anaphase chromosome spreads which are of no use for cytogenetic analysis. This treatment has proved of great value in a number of cases has no side effects; and does not induce chromosomal aberrations as far as we can determine.

Subjects

Eleven patients have been studied two patients only had typical chronic myeloid leukaemia; two others had atypical presentations. Four cases resembled subacute or acute leukaemia in their features two subjects had eosinophilic leukaemia. Brief details of these cases are given below haematologic data is assembled in Table I.

Case 1 W L. *presented with a history of tiredness, dyspnoea and aching shoulders.* On examination the spleen was enlarged 2 cm below the costal margin, and gland was enlarged in the left axilla. Blood and marrow pictures were typical of chronic mye loid leukaemia. H was treated with busulphan which caused fall in the white cell count, and reduction of spleen size. He remains in state of complete haematological remission on low maintenance dose of busulphan after 8½ years. On cessation of therapy on tw occasions he quickly relapsed. When cytogenetic studies were made the patient was in remission.

Case 2 G F presented with one year history of lassitude, cramplike abdominal pain, and subcutaneous swellings on the legs. A dental extraction 5 days prior to examination was still bleeding. *He* had no other haemorrhagic history On examination the spleen was grossly enlarged and extended into the right iliac fossa. Blood and marrow pictures were typical of chronic myeloid leukaemia. Chromosome studies were attempted without V lbe but only few mitoses were present. Treatment with busulphan reduced his white cell count and spleen size and when the white cell count was 34 000/mm³ the marrow aspiration was repeated after Velbe and satisfactory mitoses obtained.

Case 3. H. O presented with an 18 month history of tiredness and failure to concentrate. H had lost 2 stones in weight and was dyspnoeic on exertion. He had spent *many years in the Far East and had typhus in 1944 severe hepatitis in 1949–1950* and duodenal ulcer in 1958. Examination revealed liver enlarged 3 cm below the costal margin and a spleen which was minimally enlarged. At the time of presentation the patient had nonspecific uveitis and conjunctivitis. The blood picture suggested chronic myeloid leukaemia but leucocyte alkaline phosphatase was higher than normal. Marrow puncture revealed hyperplasia of the granulocyte series but with normal maturation. The paucity of mitoses prevented adequate cytogenetic study A biopsy of the liver was carried out to exclude neoplasia or amoebiasis, but apart from granulocytic infiltration no abnormality was found. Liver tomograms were also unhelpful and no abnormality was detected in the chest x-ray A diagnosis of chronic myeloid leukaemia was thought most likely and he was treated with busulphan which brought about a reduction in the white blood count and general improvement. Bone marrow puncture was performed after Velbe and study showed the presence of Ph chromosome At present he is in remission and his leucocyte alkaline phosphatase has remained normal except for one occasion when it fell to 5

Table I
Haematological data

Case	Age	Sex	Hb. g%	WBC mm³	P	L	M	B	E	EG	Blasts	Plts ×10³ p. mm³	LAP score	Date of presentation	Date of death	Treatment
					Differential count* %											
1	57	M	5.5	70,000	62	10	2	6	1	19	0	480	0	3/57	—	Busulphan
2	43	M	7.8	302,000	43	1	0	2	2	49	1	312	0	9/63	—	Busulphan
3	50	M	13.6	67,000	54	7	1	3	0	35	0	136	64	11/63	—	Busulphan
4	38	M	8.2	750,000	24	2	0	0	1	73	0	302	0	2/63	10/63	Uracil mustard Busulphan Steroids
5	52	F	5.1	9,400	61	15	1	0	0	22	1	101	0	9/62	9/63	6-mercaptopurine Prednisolone
6	23	F	6.3	450,000	42	1	0	3	3	51	0	427	0	2/64	—	6-mercaptopurine Uracil mustard Busulphan
7	45	M	7.3	45,000	40	10	5	1	0	41	3	56	42	1/64	—	Busulphan 6-mercaptopurine
8	73	M	5.1	230,000	11	12	2	0	0	68	7	122	0	2/63	9/63	6-mercaptopurine NH_4
9	49	M	9.6	4 800	30	48	1	1	1	0	19	183	0	7/64	—	Busulphan 6-mercaptopurine
10	63	M		20,200	35	7	0	0	48	10	0		62	4/64	2/65	6-mercaptopurine
11	54	M	12.2	15,100	35	10	2	0	47	8	0	106	—	6/57	—	—

E Eosinophils
P Polymorph neutrophils
L Lymphocytes
M Monocytes
B Basophils
EG Early Granulocyte

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Case 3. H. O. presented with an 18 month history of tiredness and failure to concentrate. He had lost 2 stones in weight and was dyspnoeic on exertion. He had spent many years in the Far East and had typhus in 1944, severe hepatitis in 1949–1950 and duodenal ulcer in 19-8. Examination revealed liver enlarged 3 cm below the costal margin and spleen which was minimally enlarged. At the time of presentation the patient had nonspecific uveitis and conjunctivitis. The blood picture suggested chronic myeloid leukaemia but leucocyte alkaline phosphatase was higher than normal. Marrow puncture revealed hyperplasia of the granulocyte series but with normal maturation. The paucity of mitoses prevented adequate cytogenetic study. A biopsy of the liver was carried out to exclude neoplasia or amoebiasis, but apart from granulocytic infiltration no abnormality was found. Liver tomograms were also unhelpful and no abnormality was detected in the chest -ray. A diagnosis of chronic myeloid leukaemia was thought most likely and he was treated with busulphan which brought about reduction in the white blood count and general improvement. Bone marrow puncture was performed after Velbe and study showed the presence of a Ph[1] chromosome. At present he is in remission and his leucocyte alkaline phosphatase has remained normal except for one occasion when it fell to 3.

Case 7 J W had history of 7 weeks abdominal pain, diarrhoea and dyspnoea. He had glands enlarged in his neck, both axillae and left groin liver was enlarged and the spleen was just palpable. Marrow puncture revealed granulocytic hyperplasia and was consistent with acute myeloid leukaemia and his clinical presentation was that of an acute leukaemia his blood count however suggested chronic myeloid leukaemia. Chromosome studies were then carried out before treatment. Busulphan was given with no effect, 6-mercaptopurine brought about an improvement in blood count and he has since remained well-controlled with prednisolone.

Case 8. C. F was thought to have early chronic myeloid leukaemia elsewhere when he had a white cell count of 22,400 mm³ (13% immature granulocytes) and leucocyte alkaline phosphatase of 10. Marrow puncture at that time was hyperplastic but normal. Leukaemoid reaction was suspected but search for carcinoma and intestinal parasites was fruitless. No treatment was given. When admitted in March, 1963 he had signs of prostatism, generalised lymphadenopathy and hepatosplenomegaly pain in the right leg was attributed to disc lesion. Blood count at this time showed many primitive granulocytes and the marrow puncture was consistent with acute myeloblastic leukaemia. At this time marrow was obtained for cytogenetic studies. Improvement was brought about with 6-mercaptopurine but was temporary The prostatic enlargement had given rise to troublesome symptoms and so treatment with nitrogen mustard and multiple blood transfusion was given to reduce the white cell count and improve haemoglobin levels. Transurethral prostatectomy was carried out in September 1963 and biopsy revealed adenocarcinoma. Post operatively the white cell count rose, the patient deteriorated rapidly and died with bronchopneumonia.

Case 9 S. D. had had alopecia totalis for 10 years, and 6 months before admission developed generalised rash. Marrow biopsy and blood count then were normal except for anaemia which was temporarily corrected by iron. He had been losing weight and was easily tired. On examination there were no striking physical abnormalities. Marrow biopsy revealed myeloid hyperplasia but with increased numbers of blasts and megakaryocytes. Cytogenetic studies were carried out. A diagnosis of subacute myeloid leukaemia was made and was untreated except for blood transfusion. His blood count remained unchanged for some months and then an increase in blasts occurred and busulphan was given with little effect on the blast cells, which were however reduced by 6-mercaptopurine. He has now developed chest infection and his blasts (69% in count of 21,000 WBC/mm³) are no longer controlled by 6-mercaptopurine.

Case 10. T L. presented with pains in the shoulder blades, left arm, left hip and centre back, weakness of the left leg and loss of weight. He had no detectable enlargement of lymph glands, liver or spleen. X-ray examination revealed osteoarthritic changes in cervical and lumbar spine. Marrow biopsy showed an increase in myelopoiesis and many immature eosinophils. No treatment was given. In January 1963 he developed lump on the sternum which was firm but slightly soft at the surface. Liver and spleen were now palpable. X-ray examination showed that the lump was not bony and had eroded the surface of the sternum. The white blood cell count had risen to 48,000/mm³ Needle biopsy of the tumour showed mostly primitive cells with eosinophilic granules and also some myeloblasts. *6-mercaptopurine was given but the patient died of bronchopneumonia.* The marrow t the time of death showed many myeloblasts and cells of the eosinophil series.

Case 11 R. G L. presented in 1957 with tiredness, 3 years previously he had been in hospital for treatment of empyema thoracis and at that time enlargement of liver and spleen was found and he was thought to have either leukaemia or eosinophilic granuloma. On examination in 1957 he had small glands in the cervical chain, grossly enlarged spleen, but impalpable liver H had mottled red/brown eruption over the mid-trunk and complained of intermittent pruritis for year Marrow biopsy at this time revealed

Case 7 Eleven cells were analysed. In three the Ph^1 was the only abnormality In three cells there was an additional large submetacentric chromosome, a little larger than a number 1 and with an almost terminal centromere In one of these cells a Ph^1 was present but 2 chromosomes were lacking in group 17 18. In the remaining two cells 21 22 chromosome was absent and there was no Ph chromosome, and in one the large marker chromosome was accompanied by an abnormal metacentric chromosome in the group 6–12 size range One cell was seen in which a Y chromosome was missing, but in addition chromosomes were also missing from groups 6–12 (1) 13–15 (2) and 17 18 (2) little significance can therefore be attached to the absence of the Y In two Ph positive cells small 6–12 chromosome was missing (probably No. 11 or 12) In another Ph^1 positive cell chromosome was absent in group 17 18 and there was an additional 6–12 chromosome. A cell which may have been related to the last contained two additional and identical 6–12 chromosomes and had lost 2 from group 17 18.

Case 8. In 10 cells analysed two were normal apart from the presence of the Ph^1 chromosome One hypodiploid cell had no Ph^1 and a chromosome was missing in groups 21 22 and 6–12. Four Ph-containing cells contained large submetacentric chromosome very similar to that seen in the last case and in all of these four cells small group 6–12 chromosome was absent. In one metaphase chromosomes in groups 13–15 (1) and 17 18 (2) were also absent. One Ph positive cell contained an additional 19–20 chromosome and two cells were analysed which contained an additional chromosome in groups 19–20 and 16.

Case 9 The morphology of many of the mitoses seen in this patient's marrow was poor many of the metaphase plates were fuzzy and uncountable It was very similar to the picture found in many acute leukaemia mitoses. It was however possible to analyse 6 cells. One had no Ph and was lacking in a 21 22 autosome Two Ph^1 positive cells were otherwise normal. One Ph^1 positive cell was triploid for group 2, and lacked one chromosome in groups 6–12 and 13–15. The remaining two cells showed multiple chromosome loss which was distributed throughout all chromosome groups. 2 tetraploid cells were analysable and both contained 2 Ph chromosomes.

Case 10 In this patient, studied in the terminal clinical phase mitoses were of poor quality and resembled those of acute leukaemia. 3 Ph positive cells were analysed. In all three the Ph chromosome was smaller than the group 21–22 and in one case it was clearly metacentric, the shape in the other two cells was not clear In one of these three cells the Ph^1 was the only abnormality In the second group 6–12 chromosome was absent and one acrocentric in the 6–12 size range was present. In the third cell there was an additional group 4–5 chromosome and 2 group 6–12 autosomes were missing Only 2 of the Ph negative cells were analysable; one was completely normal and the second had an extra 13–15 chromosome but lacked 2 group 17 18 and 1 group 19–20 chromosomes.

Case 11 All of the Ph positive cells analysed were otherwise normal. One of the two Ph negative cells had chromosomes missing in groups 6–12 and 13–15.

Discussion

Several points of interest and importance have emerged from the study of this series of cases. The first is the use of Velbe to increase the number of mitoses. We find that Velbe gives us better results than colchicine, which has been used by others (7) with fewer colchicine anaphase mitoses after 3 h. The Velbe does not

induce chromosomal aberration as far as we can determine. Comparing the mean percentage of modal cells in our patients who did not receive Velbe (59 /) with that in those who did (66%) we find no significant difference.

The first patient is of interest because of his long survival and good control with conventional therapy His marrow cells contained a stem-line which, as well as possessing a Ph[1] chromosome, also lacked a Y sex-chromosome. Other cases of the disease have been reported with a similar stem-line which may be associated with prolonged survival (1 19 22) The presence of such an abnormal stem line may therefore be of prognostic value and may indicate a subtype of the disease.

In the second patient with typical chronic myeloid leukaemia almost 100% of the metaphases contained a Ph[1] chromosome. His marrow at the time of study contained mitotic figures which could be identified on morphological grounds as red cell precursors. This finding would suggest that the Ph[1] chromosome is common to both erythrocyte and granulocyte precursors. Such a conclusion has also been reached by others using more refined methods for positive identification of mitotic red blood cell precursors (23 24) There is also evidence that the Ph[1] chromosome is present in cells of the megakaryocyte series. WHANG *et al.* (24) found polyploid mitoses containing the abnormality in some of their patients they could not, however exclude the possibility that these cells were either abnormal binucleate granulocytes or osteoclasts. Case 9 in the present series is interesting in this respect as his marrow at the time of study contained many megakaryocytes, and in the analysable poly ploid cells 2 Ph[1] chromosomes were present. It is clear that the Ph[1] is not present in the lymphocytic series, as lymphocyte-derived mitoses in cultures of Phytohaemagglutinin-stimulated blood leucocytes are clearly normal in patients with chronic myeloid leukaemia (6 14 24) These findings tend to indicate separate origins for lymphoid cells and for other blood cells, and is a strong argument against the hypothesis that lymphocytes can act as stem-cells for other cell types.

In Case 3 the finding of a Ph chromosome was of diagnostic importance. Here was a situation in which the blood and marrow findings were consistent with either chronic myeloid leukaemia or a leukaemoid reaction. The patient's previous history and elevated leucocyte alkaline phosphatase would certainly have suggested the

latter The low leucocyte alkaline phosphatase level has come to be accepted as one of the diagnostic criteria for chronic myeloid leukaemia and indeed many cases have a depressed enzyme level (5) Cases exist however in which the enzyme level may be normal or even elevated and in a series previously reported from this department two Ph[1] positive patients showed elevated alkaline phosphatase levels (5) KEMP *et al* (14) reported 2 patients with normal levels both had received surgical attention a few days previously which is known to cause an elevation in alkaline phosphatase levels. SPEED and LAWLER (19) reported a patient whose leucocyte alkaline phosphatase level rose from leukaemic levels after the production of marrow failure following therapy TEPLITZ *et al* (21) have reported elevated leucocyte alkaline phosphatase levels in a Ph[1] positive patient who also had ulcerative colitis and on treatment for the latter the enzyme fell to a low level. Stimuli such as operative stress and infection which may cause leucocyte alkaline phosphatase elevation in normal subjects can probably still do so in patients with typical chronic myeloid leukaemia Our patient (Case 3) had active Reiter s disease which is known to cause an elevated alkaline phosphatase level in otherwise normal subjects.

Another situation in which the leucocyte alkaline phosphatase level may rise is in blastic crisis of chronic myeloid leukaemia (5, 9). One patient in the present series (Case 7) who presented with a confusing mixture of features of both acute and chronic myeloid leukaemia and had an elevated phosphatase level may fit in this category cytogenetic studies confirmed the underlying chronic myeloid origin of his disease and also revealed new stem lines. Case 4 also showed elevation of leucocyte alkaline phosphatase levels at the time of his blastic termination. An increasing degree of aneuploidy has been reported in blastic transformation of chronic myeloid leukaemia (3 11 19) and was also a feature in our series. Case 4 had a cell line possessing two Ph[1] chromosomes such a cell line has been found in other patients in blastic crisis (11 14) and in typical chronic myeloid leukaemia Two patients in this series had cell lines containing an abnormal large, almost acrocentric chromosome (Case 7 and 8) Again similar chromosomes have been found by others but not associated with blastic crisis (12, 22) it may represent a pericentric inversion of a number 1 chromosome. Other cell lines in these patients were deficient in chromosomes in other groups and some contained additional chromosomes.

Two patients presenting with eosinophilia have been studied, one case of chronic eosinophilic leukaemia, which rapidly became acute, contained a minor Ph[1] cell line in his marrow (Case 10) whilst the more typical chronic eosinophilic leukaemia patient (Case 11) had a Ph[1] as the only abnormality in a majority of his marrow metaphases.

Previous reports about the Ph[1] chromosome in eosinophilic leukaemia have been contradictory KRAUSS *et al* (16) reported two cases of eosinophilic leukaemia, both of whom were Ph[1] negative, but KAYER and ENGLE (13) reported a case of eosinophilic leukaemia in which Ph[1] chromosome was present in circulating leucocytes.

A final word of caution is needed with regard to the Ph chromosome. Although most cases of chronic myeloid leukaemia possess the Ph[1] chromosome from the earliest stages in their history through to the terminal phase, cases do exist which are clinically and haematologically acceptable as chronic myeloid leukaemia and yet are Ph[1] negative (22) Atypical cases, bearing some of the hallmarks of chronic myeloid leukaemia have also been reported which are Ph[1] negative (11 16 19) Marrow failure as a result of cytotoxic therapy may also give rise to a transient Ph[1] negative state (19)

Three possible cases of mature cell neutrophilic leukaemia have been described, all of which are Ph[1] negative (8, 11 20) the distinction between this type of leukaemia and leukaemoid reaction is difficult to make in these cases—all had markedly elevated leucocyte alkaline phosphatase scores, and one had a small carcinoma in the lung which may have been the cause of a leukaemoid reaction (20) One further type of chronic myeloid leukaemia which is consistently Ph[1] negative although having a low leucocyte alkaline phosphatase is the juvenile form (10, 18) The absence of a Ph chromosome does not therefore exclude a diagnosis of chronic myeloid leukaemia in the presence of other features of the disease but we have found it of help in reaching a diagnosis in a number of highly atypical cases, some of them having clinical and haematological characteristics of the acute type.

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Summary

Eleven patients with chronic myeloid leukaemia have been investigated for chromosomal abnormalities of their bone marrow. Tw of these patients presented with atypical features: four cases were acute or subacute in their features and two patients had eosinophilic leukaemia. The chromosomal abnormalities found in these patients are described, the significance is discussed in the light of haematological state and prognosis.

Zusammenfassung

Bei 11 Patienten mit chronischer myeloischer Leukämie wurde das Knochenmark auf Chromosomenanomalien untersucht. Zwei dieser Patienten zeigten ein atypisches Bild. Bei 4 Fällen lagen akute oder subakute Formen vor und zwei Patienten hatten eine eosinophile Leukämie. Die Chromosomenanomalien werden beschrieben. Ihre Bedeutung im Hinblick auf den hämatologischen Status und die Prognose werden diskutiert.

Résumé

Des anomalies chromosomiques ont été recherchées dans la moelle osseuse de 11 malades atteints de leucémie myéloïde chronique. Deux de ces malades présentaient un tableau clinique atypique. Quatre cas ressemblaient à des formes aiguës ou subaiguës. Deux malades étaient atteints de leucémie éosinophile. Les anomalies chromosomiques sont décrites et leur importance par rapport au status hématologique et au pronostique est discutée.

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G 21 Trisomy in a Case of Acute Myeloblastic Leukaemia

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Whilst the presence of Ph[1] chromosome may well be the harbinger of chronic granulocytic leukaemia, no such generalisation can be made about the acute leukaemia in which there is no uniform chromosomal abnormality Indeed many cases have shown no visible aberration at all Nevertheless, there have been occasional reports of consistent abnormalities in sporadic cases. KINLOUGH and ROBSON (2) reported two cases of acute myeloblastic leukaemia (AML) with an extra abnormal chromosome in D-series. HUNGERFORD and NOWELL (1) observed in three cases of AML and WEINSTEIN and WEINSTEIN (8) in one case of AML an extra chromosome in C-series. MAKINO and SASAKI (6) presented a case of an acute myelocytic aleukaemic leukaemia showing an abnormal chromosome in C-series.

We describe here a case of AML showing G 21 trisomy in diploid and in hyperdiploid cells

Case Report

A 47 years old female was diagnosed as having AML in November 65. She had been perfectly well until the onset of the fatal illness in October 65 Physical examination disclosed signs of anaemia, erythema nodosum on the lower extremities, numerous petechiae and hepatosplenomegaly She was treated with blood transfusions, prednisone and 6-mercaptopurine. On 25th April 1966 she was referred to our clinic Clinical examination revealed anaemia, numerous petechiae enlargement of lymph-nodes and spleen 3 cm below the costal margin. Blood picture on admission white cells 53100 per mm³ (myeloblasts 86 monocytes 1% lymphocytes 13 %) Hb 12 1 g% red-cells 3.7 Mill/mm³ platelets 11200/mm³ reticulocytes o. Bone-marrow examinations on 26th April 1966 and 4th May 1966 were hypercellular with pronounced domination of myeloblasts. She was given prednisone 100 mg/day 6-mercaptopurine 150 mg/day antibiotics and blood transfusions. She died on 25th May 1966.

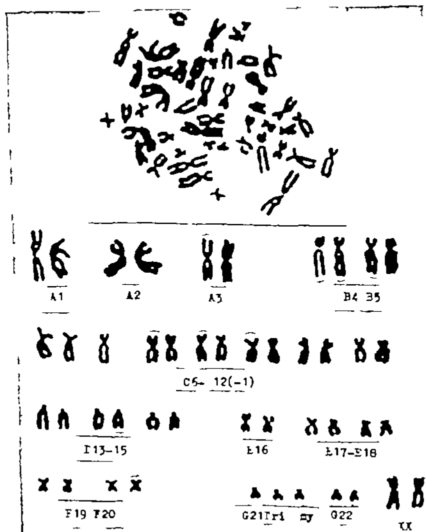


Fig. 1 Karyotype of cell line with 46 chromosomes.

Cytogenetic Analysis

Direct bone marrow chromosomal preparations were carried out on 4th May 1966, according to the method described by Kiossoglou *et al.* (3). The combined Denver (Lancet, 1960) and Patau (7) system of nomenclature was used. 40 metaphases were microphotographed and analysed. Twenty three cells had 47 chromosomes and the remaining 17 cells had 46 chromosomes. Except for one metaphase with 46 chromosomes, all other metaphases showed G 21 trisomy (Fig. 1 and 2). Karyotypes were performed on 20 cells, 13 with 47 and 7 with 46 chromosomes (Table I).



Fig. 2 Karyotype of cell line with 47 chromosomes.

Discussion

G 21 trisomy is known to occur in Down's Syndrom (3). In some cases of AML G 21 trisomy has been described in some hyperdiploid cells, which were accompanied by other multiple aberrations (4). In the case presented two different stemlines were present, characterised by chromosome number 47 and 46 respec

Table I

Cells karyotyped	Chromosome No.	Affected series PATAU nomenclature A	B	C	D	E	F	G	Comments
13	47							+1	trisomy G 21
7 {	46			—1				+1	trisomy G 21
2	46					—1		+1	trisomy G 21
2	46						—1	+1	trisomy G 21

tively To our knoweldge such a high frequency of G 21 trisomy in diploid and in hyperdiploid cells of AML have not been described. Buccal smears showed a normal female pattern. Since our patient did not show any mental or physical abnormalities other than related to leukaemia, it is very probable that the G 21 trisomy is allied to the haematological disorder Further studies of similar cases may tell us something about the aetiology of leukaemia.

Summary

G 21 trisomy is reported in female with acute myeloblastic leukaemia. Two stemlines with chromosome number 47 and 46 were present. G 21 trisomy was consistently found in both stemlines.

Zusammenfassung

Es wird über eine G 21 Trisomie bei einer Patientin mit akuter Myleoblastenleukämie berichtet. Es fanden sich 2 Generationsreihen mit 47 und 46 Chromosomen. Die G 21 Trisomie wurde übereinstimmend in beiden Reihen nachgewiesen.

Résumé

U cas de trisomie G 21 chez une femme atteinte de leucémie myéloblastique aiguë est rapporté Deux lignées de cellules comptant 47 et 46 chromosomes étaient présentes. La trisomie G 26 put être démontrée dans les deux lignées.

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Zur Struktur des Bence-Jones-Proteins*

O. Wetter, W. Braun und Ch. Hertenstein

Als Analoga der in allen Immunglobulinen (IG) vorkommenden leichten Polypeptidketten oder L-Ketten (Mol.-Gew. 20000) haben die Bence-Jones-Proteine (B.-J.-Protein) in den letzten Jahren zunehmendes Interesse gefunden. Zum Zustandekommen der Antikörperwirksamkeit von IG sind L-Ketten ebenso wie die schweren Polypeptidketten oder H-Ketten (Mol.-Gew. 50000) erforderlich (1, 2, 3). γG-Proteine mit spezifischer Antikörperwirksamkeit gegen verschiedene Haptene weisen eine unterschiedliche Aminosäurezusammensetzung auf, die nicht auf die Zugehörigkeit zu verschiedenen genetischen Typen des IG-Moleküls zurückzuführen ist (4). Isolierte Paraproteine vom γG-, γA- und γM-Typ weisen dagegen keine Unterschiede in der Brutto-Aminosäurezusammensetzung gegenüber normalen γG-Proteinen auf (5). Die Unterschiede spezifischer Antikörper betreffen sowohl die L-Ketten als auch die H-Ketten (6) und sind individualspezifisch. Auch B.-J.-Proteine besitzen Individualspezifität (7, 8). Abgesehen von dieser individuellen Verschiedenheit lassen sich die B.-J.-Proteine auf Grund ihrer Antigenstruktur in zwei Gruppen einteilen (9), die als Typ I und Typ II oder K und L bezeichnet werden. Plasmozytom-Patienten scheiden gewöhnlich B.-J.-Proteine des Typs aus, den das Paraprotein im Serum des betreffenden Patienten besitzt (10, 11).

Die Bemühungen um Aufklärung der strukturellen Grundlagen der Antigen-Antikörperreaktion gingen einmal von der Frage nach der Primärstruktur der in die Reaktion einbezogenen molekularen Bezirke des IG-Moleküls aus und zum anderen von der während der Reaktion auftretenden Konformationsänderung. Da es möglich

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erscheint, daß B.-J.-Proteine mit den L-Ketten spezifischer Antikörpermoleküle in der Primärstruktur übereinstimmen, wurde die Aminosäuresequenz verschiedener B.-J.-Proteine untersucht, um daraus Aufschluß über die strukturellen Grundlagen der Antikörperspezifität und möglicherweise über den genetischen Mechanismus der L-Kettensynthese zu gewinnen. Derartige Untersuchungen sind von mehreren Arbeitsgruppen (12, 13, 14, 15, 16) begonnen worden, so daß z. Zt. die Primärstruktur von 3 B.-J.-Proteinen (Roy, Ag, Cum) vom Typ I weitgehend bzw. teilweise bekannt ist (17, 18). Es stellte sich heraus, daß weitgehende Übereinstimmung der Aminosäuresequenz der carboxylendständigen Hälfte des B.-J.-Proteinmoleküls der 3 Fälle besteht. Deutliche Variabilität dagegen weist die Primärstruktur der aminoendständigen Hälfte auf. Die Ursachen dieser Variation sind von Stevens (19) diskutiert worden. Das Peptidmuster von Typ K (I)- und Typ L (II)-B.-J.-Proteinen ist völlig verschieden voneinander, während B.-J.-Proteine des gleichen Typs eine Reihe von Peptiden gemeinsam haben, daneben aber auch eine individuelle Variabilität des Peptidmusters aufweisen (20). Es gelang bei 3 B.-J.-Proteinen eine die individualspezifischen Peptide enthaltende Untereinheit von der die gemeinsamen Peptide enthaltenden Komponente zu trennen (21).

Untersuchungen über die Dimensionen eines B.-J.-Proteinmoleküls mittels der Röntgen-Kleinwinkelstreuung ergaben, daß es sich um ein globuläres Molekül mit den Achsen 21,0 Å, 48,3 Å und 74,8 Å bei einem Mol.-Gew. von 45000 handelte (22). Durch Chromatographie eines elektrophoretisch einheitlichen B.-J.-Proteins an DEAE-Ionenaustauscher konnte eine Fraktionierung in 3 Komponenten erreicht werden, die sich in ihrer Aminosäurenzusammensetzung nur leicht unterscheiden. Die spezifische Drehfähigkeit dieser Komponenten dagegen wies keine Unterschiede auf (23).

Die Ursache der auch in der Stärkegelelektrophorese beobachteten Heterogenität von B.-J.-Proteinen konnte auf Dimerisierung bzw. Polymerisierung zurückgeführt werden (24). Die einzelnen Komponenten wiesen keine Unterschiede der Aminosäurenzusammensetzung, der Peptidmuster und der Antigenstruktur auf. Eine Beziehung zwischen dem immunologischen Typ eines B.-J.-Proteins und seinem physikochemischen Verhalten scheint insofern zu bestehen, als B.-J.-Proteine vom Typ K (I) gewöhnlich als Monomere vorkommen, während B.-J.-Proteine vom Typ L (II) überwiegend als Dimere oder Polymere existieren (25). Dimerisation kann durch leicht dissoziable nicht-kovalente Kräfte oder durch eine Disulfidbrücke in stabiler Form hervorgerufen werden (26). Durch sedimentationsanalytische, viskosimetrische und polarimetrische Untersuchungen unter Harnstoffeinwirkung auf B.-J.-Proteine konnte darüber hinaus die Bedeutung nicht kovalenter, wahrscheinlich hydrophober Bindungen für die Konformation von B.-J.-Proteinen beider Typen gezeigt werden (27).

Die nachstehend mitgeteilten Befunde sollen zum Verständnis der strukturellen Beziehungen zwischen B.-J.-Proteinen und IG beitragen. Die Bedeutung der optischen Rotationsdispersion für die Strukturanalyse von B.-J.-Proteinen wird kritisch untersucht.

Methoden und Material

B.-J.-Proteine wurden aus dem Harn von Plasmocytom-Patienten durch Erhitzen bei 60° C und mehrfache Umfällung (H.-Ba.) oder chromatographisch an DEAE-Sephadex A 50 nach Ultrafiltration des Urins (Be., Hb., Ka., Mo., Sp.) gewonnen. Die Präparate wurden elektrophoretisch, sedimentationsanalytisch, immunologisch und in der Stärkegelelektrophorese rein erachtet.

Die Sedimentationsanalysen wurden in der analytischen Ultrazentrifuge Spinco Modell E bei 59 780 UpM und 20 °C vorgenommen. Die Proteinkonzentration lag zwischen 1,5 und 15 mg/ml. Als Puffer diente Veronalpuffer pH 8,6, Ionenstärke 0,1. Es erfolgte keine Korrektur der Sedimentationskoeffizienten auf Dichte und Viskosität von Wasser bei 20 °C.

Die Immunelektrophorese wurde nach der Vorschrift von SCHEIDEGGER (28) in 2%igem Agar der Behring-Werke durchgeführt. Als Antiseren wurden käufliche Antihumanseren der Behring-Werke und selbst hergestellte Antiseren von Kaninchen benutzt. Die Typendifferenzierung der B.J. Proteine erfolgte in Agarplatten nach OUCHTERLONY mit Anti-K (I)-Antiseren und Anti-L (II)-Antiseren der Fa. Hyland/Los Angeles.

Bei der Stärkegelelektrophorese wurde nach der von SMITHIES (29) angegebenen Methode vorgegangen. Die Harnstoff-Stärkegelelektrophorese wurde nach EDELMAN und POULIK (30) vorgenommen.

Für die Säulenchromatographie wurde DEAE Zellulose Anionenaustauscher Sephadex A 50 der Fa. Pharmacia, Uppsala, verwendet. Die Elution erfolgte schrittweise mit Phosphatpuffern steigender Ionenstärke bei pH 7,0 und 6,0, beginnend mit einem 0,008 m Phosphatpuffer (31). Das Eluat wurde automatisch fraktioniert (RadiRac der LKB Producter, Stockholm) und im Uvicord registriert. Die für weitere Untersuchungen benutzten Fraktionen wurden mittels der vorstehend angegebenen Methoden getestet. Außerdem wurden diese Proben trägerelektrophoretisch auf Acetatfolie der Fa. Schleicher und Schüll, Dassel, im Hochspannungsgerät nach WIELAND und PFLEIDERER (Fa. Hormuth, Wiesloch) in Veronalpuffer pH 8,6 bei 30 V·cm⁻¹ und zirka 60 Min. Laufzeit untersucht.

Für die Messungen der optischen Rotationsdispersion (ORD) wurde das lichtelektrische Präzisionspolarimeter LEP 0,005 der Fa. Zeiss, Oberkochen, benutzt. Es wurde bei Zimmertemperatur und in der 5 cm Küvette gemessen. Die Dispersionskonstante λ_c und die optischen Parameter a_0 und b_0 wurden nach den früher beschriebenen Verfahren ermittelt (32,33).

Die Bestimmung des Proteingehaltes der Lösungen erfolgte mit dem Biuretreagenz nach WEICHSELBAUM in der von BEISENHERZ (34) angegebenen Modifikation.

Für die Peptidanalysen wurden säulenchromatographisch isolierte B.J. Proteine lyophilisiert und bis zur Verarbeitung bei 4 °C aufbewahrt. Nach dem Vorgehen von SCHWARTZ und EDELMAN (35) wurden 10 mg Protein in 1,4 ml 8 m Harnstoff mit 0,1 m Merkaptoäthanol aufgenommen. Nach 4 Std. wurde die Lösung mit Jodacetamid bis zu einer Konzentration von 0,2 m Jodacetamid versetzt und 10 Min. bei Raumtemperatur belassen. Anschließend erfolgte eine ausgiebige Dialyse gegen 0,2 m NH_4HCO_3 bei 4 °C. Die tryptische Hydrolyse wurde über 23 Std. bei 37 °C mit 2 μg/mg Trypsin der Fa. Serva, Heidelberg, durchgeführt. Das Hydrolysat wurde jeweils gefriergetrocknet und in dem für die Elektrophorese benutzten Pyridin-Eisessig-Wasser-Puffer (50:5:445) aufgenommen. 0,01 ml des Hydrolysats entsprechend ca. 1 mg Protein wurden auf vorbereitete Platten zur Dünnschichtchromatographie (Fa. Desaga, Heidelberg) in MN Kieselgel S (Fa. Macherey, Nagel und Co.) aufgetragen. Damit weicht unser Vorgehen von demjenigen der zitierten Autoren (35), die mit Whatman 3 MM Papier arbeiteten, wesentlich ab. Die Elektrophorese erfolgt in dem für die Trägerelektrophorese benutzten Gerät bei 5 °C, 35 V·cm⁻¹ über etwa 45 Min. Anschließend wurde die Chromatographie in der zweiten Dimension vorgenommen. Als Fließmittel wurden 1.) Butanol-Eisessig-Wasser im Verhältnis 4:1:5 und 2.) Chloroform-Methanol-Ammoniak im Verhältnis 2:2:1 benutzt. Die Entwicklung der Peptidflecken erfolgte mittels Ninhydrin-Sprühreagenz.

Herrn Prof. Dr. G. PFLEIDERER und Mitarbeiter, Frankfurt/M., danken wir für wertvolle methodische Hinweise.

Ergebnisse

Charakteristische Eigenschaften der für die Messungen der ORD verwendeten B J Proteine sind in Tabelle I zusammengefaßt. Die Proben Ba, He und Hh scheinen überwiegend in der monomeren Form vorzuliegen Es ist ersichtlich, daß keine Beziehung zwischen der elektrophoretischen Beweglichkeit und dem immunologischen Typ eines B J Proteins besteht. Das B.J Protein mit dem niedrigsten Sedimentationskoeffizienten (Monomer) der hier untersuchten Proben (Hh) weist chromatographisch keine derart ausgesprochene Heterogenität auf wie die übrigen chromatographisch untersuchten B J Proteine vom Typ I Stärkegelelektrophoretisch sind auch die aus der Mitte der Gipfel des Elutionsdiagramms entnommenen chromatographisch sicher homogenen Proben heterogen (Tabelle I rechte Spalte) In Abb. I ist das Original der Stärkegelelektrophorese von 5 chromatographisch isolierten B J Proteinen wiedergegeben.

Das Elutionsdiagramm des Harn-Ultrafiltrats Sp mit 0.008 m Phosphatpuffer ist in Abb 2 zugleich mit dem Ergebnis der immunelektrophoretischen und sedimentationsanalytischen Untersuchung des Eluats wiedergegeben. Aus der Abbildung geht hervor daß zwischen den immunologisch als B J Protein identifizierten

Tabelle I

Charakterisierung zweier hitzegefällter und 5 chromatographisch isolierter B. J. Proteine.

Probe	Typ	Präparation	S_{20}	Elektrophoretische Beweglichkeit	Chromatographische Kompon.	Stärkegelelektrophorese Kompon.	Schema
Ba	K (I	Hitzefällung	3.2	β			
He	K (I)	Hitzefällung	3.2*	α2			
Be	K I	Chromatographie	3.7	[illegible]	3	2	
Hh	K (I	Chromatographie	2.6	γ	3	3	
Mo	K (I	Chromatographie	3.5	γ	3	4	
Sp	K (I	Chromatographie	3 4	γ	3	3	
Kn	L (II	Chromatographie	4.02	γ	4	4	

* Sedimentationskonstanten

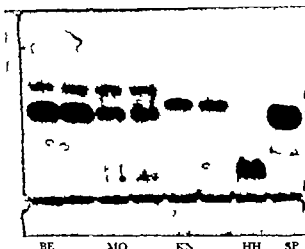


Abb. 1 Harnstoff-Stärkegelelektrophorese 5 chromatographisch isolierter B. J. Proteine

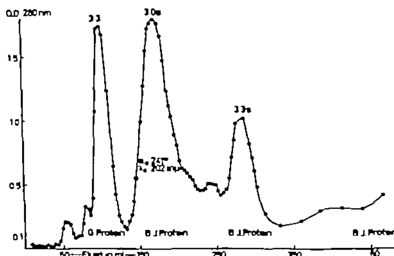


Abb. 2. Elutionsdiagramm eines Harn-Ultrafiltrats bei Bence Jones-Proteinurie (Sp) mit 0,008 m Phosphatpuffer pH 7,0 bei 4° C. Austauscher DEAE Sephadex A 50.

Fraktionen des zweiten und dritten Gipfels kein wesentlicher Unterschied der Sedimentationskoeffizienten besteht. Der erste Gipfel des Diagramms Sp repräsentiert ein leichtes γG-Protein (36). Die vor dem ersten und zwischen dem zweiten und dritten Gipfel sichtbaren Komponenten konnten wegen zu geringer Konzentration nicht identifiziert werden. Prinzipiell ähnlich wie im Fall Sp waren die Elutionsdiagramme der Fälle Be, Kn und Mo.

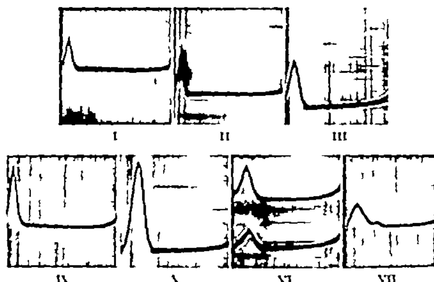


Abb. 3. Sedimentationsdiagramme 6 chromatographisch und eines durch Hitzefällung H... isolierten Bence Jones-Proteins. Aufnahmezeiten in Minuten nach Erreichen der Höchstgeschwindigkeit: I (Ba.) 17, II (Be.) 35, III (He.) 26, VI (Hb.) 37, V (Ka.) 12, VI Mo., VII Sp. 23. Sedimentationsrichtung von links nach rechts.

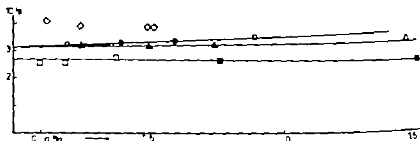


Abb. 4. Konzentrationsabhängigkeit der Sedimentationskoeffizienten der 4 Bence-Jones-Proteine

Be.
Ba.
He.
Hb.

in Veronalpuffer Ionenstärke 0,1, pH 8,6 bzw. 0,008 m Phosphatpuffer pH 7,0 (Hb.) bei 20° C.

In Abb. 3 sind die Sedimentationsdiagramme der B.J.-Proteine wiedergegeben. Mit Ausnahme der Probe Hb handelt es sich um einheitliche Gradienten, deren Sedimentationskoeffizienten in dem für B. J. Proteine charakteristischen Bereich von 3 bis 4 s liegen. In 4 Fällen wurde die Konzentrationsabhängigkeit der Sedimentationskoeffizienten untersucht. Das Ergebnis ist in Abb. 4

Tabelle II

Dispersionskonstanten und optische Parameter a_0 und b_0 nach Moffitt und Yang an 7 isolierten B. J Proteinen.

Probe	Typ	Konzentration mg/ml	Puffer	λ_c nm	$-a_0$	b_0	α-Helix %
Ba	K (I)	5.2	Veronalpuffer pH 8 6	218	240	−22.4	3.6
He	K (I)	7.9	Veronalpuffer pH 8 6	217	338	13.1	2.1
Be	K (I)	15.0	Veronalpuffer pH 8.6	198	171	25.9	0
Hh	K (I)	4 7	Veronalpuffer pH 8.6	214	244	5 8	0
M	K (I)	1 4	0 008 m Phosphatpuffer	229	263	23.3	3.7
Sp	K (I)	6.3	Veronalpuffer pH 8.6	202	241	19 1	0
Kn	L (II)	3.0	Veronalpuffer pH 8 6	196	125	14.9	0

dargestellt. Alle Proben gehören zum Typ K (I) Während die B J Proteine Ba, Be und He eine lineare Abhängigkeit aufweisen weicht Hh in seinem Sedimentationsverhalten deutlich von den vorgenannten Proben ab

Die Ergebnisse der Messungen der ORD sind in Abbildung 5 dargestellt und in Tabelle II zusammengefaßt. Die Dispersionskonstante λ_c liegt mit einer Ausnahme (Mo) in dem für alle IG charakteristischen Bereich. Die Ursache des relativ hohen Wertes von 229 nm bei Mo ist nicht ersichtlich. Die Konstante b_0, aus der sich der α Helixgehalt eines Proteins angenähert bestimmen läßt, besitzt Werte, die nicht stark von 0 abweichen Ein nennenswerter Helixgehalt läßt sich hieraus nicht entnehmen. Beträchtliche Unterschiede weist dagegen der Parameter a_0 auf. Er liegt zwischen 125 und 338 In Abb 6 sind die a Werte zugleich mit denen normaler und pathologischer γG-Proteine sowie pathologischer γM Proteine graphisch aufgetragen Die beobachteten individuellen Unterschiede von a innerhalb der B J Proteine lassen sich nicht auf elektrophoretische, chromatographische oder sedimentationsanalytische Besonderheiten zurückführen Es wurden deswegen die Peptidmuster nach tryptischer Spaltung chromatographisch isolierter B. J Proteine untersucht

In Abb. 7 ist als Beispiel einer derartigen Peptidanalyse das mit dem B J Protein Hh erhaltenen Muster vor und nach Mar

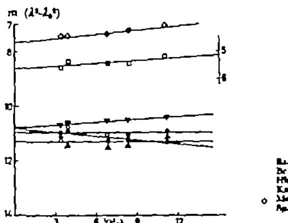


Abb. 5. Optische Rotationsdispersion 6 chromatographisch isolierter B. J. Proteine nach Moffitt und Yang in Veronalpuffer, Ionenstärke 0,1, pH 8,6 (Probe Mo. in 0,008 m Phosphatpuffer pH 7,0)

kierung der Flecken durch Umrandung wiedergegeben. Es lassen sich 35 Flecken dieses B. J. Proteins vom Typ K (I) differenzieren. Ein Vergleich der von den Proben Hh, Ba, Be und Sp gewonnenen Peptidmuster läßt bei guter Reproduzibilität eine deutliche Variabilität innerhalb dieser zum gleichen immunologischen Typ (K oder I) gehörigen B. J. Proteine erkennen. Sie ist Ausdruck der Individualspezifität dieser Proteine. Die prinzipielle Verschiedenheit der Peptidmuster zweier B. J. Proteine vom Typ K (I) und L (II) geht aus der Abb. 8 hervor. Hier sind die Chromatogramme eines B. J. Proteins vom Typ K (I) und vom Typ L (II) einzeln und nach Mischung der durch tryptische Hydrolyse erhaltenen Peptide, d. h. kombiniert untersucht. Man erkennt, daß die Kombination fast der Summe der Peptide aus dem Typ K- und Typ L-B. J. Protein entspricht. B. J. Protein Kn und Be besitzen demnach kaum ein gemeinsames Peptid.

Diskussion

Ebenso wie bei den IG vom γG-, γA- und γM-Typ wird die globuläre Struktur der B. J. Proteine durch Disulfidbrücken zwischen H- und L-Kette und durch nicht kovalente Kräfte stabilisiert. Zwischen B. J. Proteinen vom Typ K (I) und L (II) besteht kein Unterschied bezüglich der durch Messungen der

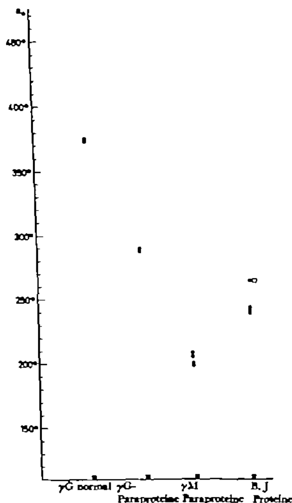


Abb. 6. Optischer Parameter $-a_0$ nach Moffitt und Yang von 7 γG Proteinen Gesunder 20 γG Paraproteinen, 17 γM Paraproteinen und 7 Bence Jones Proteinen. Werte nach Migita (46) durch Quadrate bezeichnet.

Viskosität und des Verhaltens bei der Harnstoffdenaturation bestimmbaren Konformation (27). Dagegen scheinen sich B J Proteine vom Typ K und Typ L in ihrem Sedimentationsverhalten (25) und in ihrer UV Absorption in Gegenwart von Harnstoff oder Guanidinhydrochlorid zu unterscheiden (37). Insbesonders der

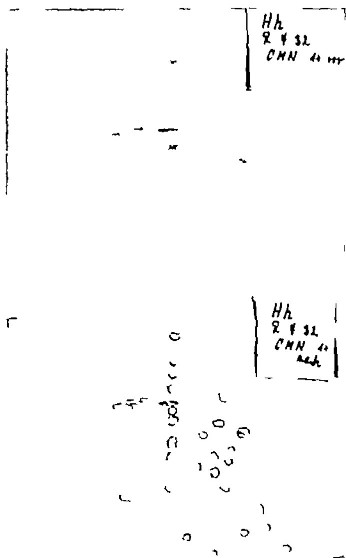


Abb. 7 Peptidanalyse des B. J. Proteins Hh. (Typ κ) nach tryptischer Hydrolyse und Hochspannungselektrophorese bei pH 6.5 in horizontaler Richtung (Kathode rechts), Chromatographie in vertikaler Richtung. Position der Pikrinsäuremarkierung nach Chromatographie durch Winkelzeichen oben links angedeutet.

letztere Befund ist mit unseren an anderer Stelle (27) mitgeteilten Beobachtungen schlecht in Einklang zu bringen.

Die in den vorausgehenden Mitteilungen von uns beschriebenen Unterschiede der ORD von isolierten γG-(32) γM (33) und γA (38) Paraproteinen wurden auf spezifische Strukturen bezogen,

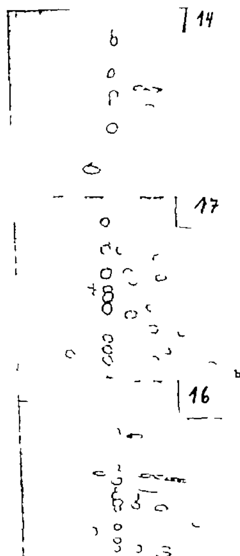


Abb. 4. Peptidanalyse eines B.J. Proteins vom Typ K (oben) und vom Typ L (mitte) und nach Mischung beider tryptischen Hydrolysate (unten). Elektrophorese in horizontaler Richtung (Kathode rechts). Chromatographie in vertikaler Richtung

die für IG vom γG, γA und γM Typ charakteristisch sind. Die beobachteten Unterschiede betrafen die optische Konstante a_0. Diese setzt sich aus den Komponenten a^H und a_0^R zusammen. a^H ist vom Ausmaß des α-Helixgehaltes oder einer anderen perio-

die Typenspezifität des a_0 Wertes von γG- γA- und γM Paraproteinen scheint der Einfluß der betreffenden schweren Polypeptidketten auf das optische Drehvermögen zu sein. Die optische Konstante-a_0 bildet demnach eine Entsprechung zu den immunologischen Eigenschaften der 3 großen Immunglobulin-Typen.

Summary

The optical parameter -a_0 of isolated Bence Jones proteins show considerable individual variability. These results of measurements of the optical rotatory dispersion is in accord with the results of peptide mapping, chromatographic and starch gel electrophoretical analyses. The observed differences are caused by the different primary structure of these the L-chains of normal immunoglobulins resembling proteins. Individual specifity of the optical constant-a_0 has been found in isolated paraproteins of the γG- γA- and γM-type as well. These individual differences however are superimposed by type specific differences between the γG-, γA- and γM-immunoglobulins. Deciding for the type specifity of a_0 of paraproteins is most probably the influence of H-chains on the optical rotatory dispersion.

Résumé

La détermination du paramètre optique a_0 de 7 protéines de Bence Jones isolées mit en évidence une grande variabilité individuelle. Celle-ci correspond aux résultats des analyses peptidiques, de la chromatographie et de l'électrophorèse en gel d'amidon. Elle est due aux différences de la structure primaire de ces protéines ressemblant aux chaînes L des immunoglobulines normales. Des différences individuelles de la constante optique a_0 purent être aussi démontrées chez les paraprotéines. A celles-ci se superposent cependant des différences spécifiques à leur type, différences qui semblent être dues à une influence décisive des chaînes peptidiques lourdes H des immunoglobulines γG, γA et γM.

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Division of Leukocytes Already in DNA Synthesis from Patients with Acute Leukemia and Infectious Mononucleosis

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Atypical lymphocytes in infectious mononucleosis have been considered to be highly differentiated mature cells (1). We recently showed (2) that atypical lymphocytes of patients with infectious mononucleosis included a population of actively dividing cells. These cells differed from normal resting cells. They had been induced to proliferate by a stimulus *in vivo* and divided within 8 h *in vitro*. Normal resting lymphocytes, on the other hand, required a mitotic stimulant such as phytohemagglutinin *in vitro* and about 40 hours' incubation before mitosis began. This study compares the rate of division of cells stimulated to divide *in vivo* in infectious mononucleosis with a similar population in acute leukemia.

Material and Methods

Eleven patients with acute leukemia in relapse were seen at the Veterans Administration Hospital, or the University of Wisconsin Hospitals. The diagnosis was based on typical history, physical findings and cytological study. In one case the cell type was not certain. Some patients were being treated at the time of study. Only 3 patients were alive at the conclusion of the study. The clinical data for these patients are given in Table I.

Fourteen patients with typical history, physical findings and laboratory data of infectious mononucleosis were referred from the Student Infirmary of the University of Wisconsin Hospitals. The peripheral blood of all of these patients had more than 20% atypical lymphocytes within 3 days of study.

Peripheral blood was mixed with heparin (10 mg/ml) 1 ml/30 ml of blood. Red cells settled spontaneously within 30–45 min. Supernatant plasma containing leukocytes was divided into 2 culture bottles so that each bottle contained 2 ml of plasma and cells, and 8 ml of tissue culture medium 199. Colchicine in final concentration of 1×10^{-6} M was added to 1 aliquot and tritiated thymidine* 0.5–1.0 μc/ml to the other. No mitotic

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* 1.9 C/mmol

Table I
Clinical data of patients with acute leukemia.

[illegible]ent	Disease	Drug	Daily dose	Drug	Daily dose	Age	Status	Remarks
[illegible]	ALL	6-mercapto-purine	200 mg	Prednisone	15 mg	25	D	WBC [illegible] [illegible]% bla[illegible]
[illegible]	AML+					65	D	WBC [illegible] 6% blas[illegible]
[illegible]	AL×	cytoxan	25 mg			2	D	WBC [illegible]
	ALL					20	L	WBC [illegible] 73% bla[illegible]
	ALL	methotrexate	50 mg	6-mercapto-purine	75 mg	7	D	WBC [illegible] [illegible] bla[illegible]
	AML					11	D	WBC [illegible] 15% bla[illegible]
	ALL	6-mercapto-purine	200 mg (1 day)	Prednisone	40 mg (1 day)	41	D	WBC [illegible] [illegible] bla[illegible]
	AML					68	D	WBC [illegible] [illegible] pro[illegible]
[illegible]	AML					9	L	WBC [illegible] [illegible]% pro[illegible]
	AML					69	D	WBC [illegible] 5% bla[illegible]
[illegible]	ALL					4	L	WBC [illegible] 70% bla[illegible]

[illegible]te lymphocytic leukemia
[illegible]te myelogenous leukemia
[illegible]te leukemia type unspecified
[illegible]d
[illegible]g

stimulants were added. The final concentration of white cells was 1/5th the concentration in plasma.

Mitoses were allowed to accumulate for 4–8 h at 37 °C. Air-dried metaphase preparations were made after fixation with acetic alcohol. Three thousand to 10,000 cells were counted for estimation of mitotic activity. If no mitoses were seen in 10,000 cells, the proportion was expressed as less than 1/10,000. The per cent mitosis was divided by the hours of incubation in colchicine in order to obtain the mitotic rate per hour.

After the second aliquot was exposed to tritiated thymidine 2–4 h, coverslip smears were made and processed for autoradiography using AR 10 stripping film. The slides were exposed at 4 °C for 7 days and stained with Giemsa after development. One thousand cells were counted from each preparation and tabulated as per cent labeled cells or per cent cells in DNA synthesis. In 3 studies there was no significant increase in proportion of labeled cells after 2 h. The per cent labeled cells were therefore not divided by the hours of incubation. Average grain counts were made by counting silver grains of 50–100 consecutive labeled cells. Densely labeled cells were scored as greater than 100 grains. Since most of the cells were highly labeled, the per cent cells with more than 100 grains was used as a numerical index.

Results

The mean mitotic rate was 0 08% per hour exposure to colchicine (range 0 01–0 18%) for infectious mononucleosis cultures The mean proportion of cells labeled with tritiated thymidine in infectious mononucleosis was 7 6% (range 1 7–19.5%) The mean ratio of per cent labeled cells per per cent dividing cells was 123 (range 46–300) indicating that 1 cell would divide per hour for every hundred infectious mononucleosis cells in DNA synthesis in this system.

The mean mitotic rate for acute leukemia cells was less than 0.003% per hour No mitoses were seen in 5 of 11 studies. The mean proportion of labeled cells was 2.4% (range 0.3–6.1%) There was no correlation between the proportion of blast cells and the proportion of cells incorporating tritiated thymidine. The mean ratio of per cent labeled cells per per cent dividing cells was greater than 1027 (range 270–more than 2900) This suggests that less than 1 cell would divide per hour for every thousand acute leukemia cells in DNA synthesis. Fig 1 shows the per cent labeled cells plotted

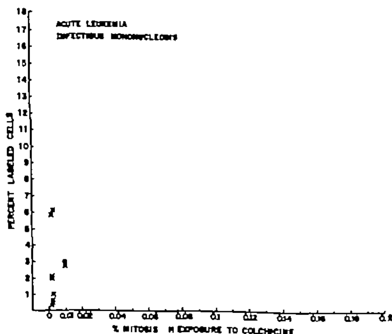


Fig 1 Comparison between proliferative indices of acute leukemia and infectious mononucleosis.

against the per cent dividing cells per hour for both diseases. Fig 2 shows the rate of appearance of metaphases in 6 patients with infectious mononucleosis. Metaphases were seen at 1 h in each case. Metaphases in acute leukemia were too rare to be plotted for periods less than 6 h.

The mean proportion of labeled cells with more than 100 grains was higher in infectious mononucleosis (88% range 70–98%) than in acute leukemia (76% range 44–89%) The p value for a difference between the two groups was less than 0.05 by the t test.

Discussion

The study indicates that for every 1000 cells in DNA synthesis, less than one peripheral acute leukemia cell divides per hour *in vitro* *In infectious mononucleosis, 1 cell divides per hour for every* hundred cells in DNA synthesis. Although proliferation in acute leukemia is almost certainly slow we cannot be certain that the rate of cell division is normal in infectious mononucleosis. In normal lymphocytes stimulated to divide by phytohemagglutinin, 1 cell is

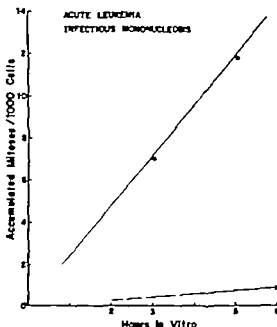


Fig 2. Appearance of mitoses in cultures of infectious mononucleosis and acute leukemia treated with colchicine at time zero. Normal cells cultured with colchicine six hours show no mitoses. Acute leukemia cells averaged one mitosis/1000 at the end of 6 h. Each point represents the mean of 4–6 experiments.

in division per hour for every 40 in DNA synthesis. Infectious mononucleosis peripheral leukocytes divide more slowly than these normal cells and at the same rate as peripheral leukocytes from chronic myelocytic leukemia*

Previous studies have suggested a slow rate of cell division in acute leukemia (3–6) This might be explained by a prolonged G_2 (premitotic, post DNA synthesis) period. The rate of cell division did not increase over a 24-hour period however so that this explanation requires a G period greater than the usual total generation time. Another explanation would be a prolonged S (period of DNA synthesis) time. In that case, less tritiated precursor should be incorporated per unit time and grain counts should be lower in short incubation. Our data showing lower grain counts in acute leukemia confirm those of LAJTHA (1) and CRADDOCK and NAKAI (8)

It is doubtful, however that thymidine grain counts are a reliable index of the rate of DNA synthesis. Exogenous thymidine circumvents the marked reduction of thymidylate synthetase in acute leukemia (9) This enzyme catalyzes a rate-limiting step in DNA synthesis. Consequently we cannot prove from thymidine incorporation that DNA synthesis is prolonged. HALE and COOPER (10) concluded that DNA synthesis follows a similar pattern in acute leukemia and infectious mononucleosis. We have no explanation for the slow proliferation in acute leukemia.

No direct comparisons of leukocyte proliferation as described here have been reported Our previous study of mitotic rate in infectious mononucleosis has been confirmed (11) In view of the number of cells in division as early as 1 h after incubation with colchicine the rarity of mitotic figures in peripheral blood is surprising Probably these cells have a short transit time in the circulation in contrast to leukemic cells (12)

This study was done by simple methods on peripheral blood. The techniques are distinct improvement over morphologic interpretation of blood smears. Such studies are helpful in our hands in evaluating a patient with prolonged serious illness resembling infectious mono- without serologic confirmation. Whether the tests will prove to be of general clinical value requires further evaluation.

Acknowledgment. The work of YVONNE HOMSTAD and MARGARET HOLLAND made this study possible

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* unpublished data

Summary

Short term incubation of acute leukemia cells indicates that for every 1000 cells which incorporate tritiated thymidine, fewer than 1 divides per hour In the same system, 1 atypical lymphocyte from infectious mononucleosis divides for every 100 cells in DNA synthesis. These data support the idea that cell division is slow in acute leukemia, and that, in contrast, cells are rapidly proliferating in infectious mononucleosis.

Zusammenfassung

Kurzfristige Inkubation von Zellen akuter Leukämien zeigt, daß auf 1000 Zellen, die Tritium-markiertes Thymidin aufnehmen, weniger als eine pro Stunde sich teilt. Im gleichen System teilt sich ein atypischer Lymphocyt der Mononucleosis infectiosa pro 100 Zellen mit DNA-Synthese. Diese Ergebnisse stützen die Auffassung, daß die Zellteilung bei akuter Leukämie langsam verläuft und daß im Gegensatz dazu die Zellen bei der Mononucleosis infectiosa rasch proliferieren.

Résumé

L'incubation de courte durée de cellules de leucémies aiguës montre que sur 1000 cellules ayant incorporé de la thymidine tritiée, moins d'une se divise en l'espace d'une heure. Dans le même système, un lymphocyte atypique de la mononucléose infectieuse seulement sur 100 cellules synthétisant de l'ADN se divise. Ces résultats concordent avec l'idée que dans la leucémie aiguë, la prolifération cellulaire est lente et qu'elle est en revanche, rapide dans la mononucléose infectieuse.

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In vitro Study of DNA Synthesis Time and Cell Cycle Time in Erythrocyte Precursors of Normal and Thalassaemic Subjects, Using a ^{3}H and ^{14}C-Thymidine Double Labelling Technique

M. Kesse Elias, E. B. Harris and E. Gyftaki

In spite of the known inheritable defects in haemoglobin synthesis in thalassaemia the exact nature of the abnormality of erythropoiesis is, as yet, still obscure. As a contribution towards this problem we are investigating the rate of marrow cell proliferation in thalassaemic subjects. The data are also of interest for the comparison of thalassaemia with other haematological disorders. The present paper gives some preliminary results in 9 thalassaemic and 5 normal subjects.

Since DNA synthesis occurs during a specific interval in the cell cycle (the S-phase) (9) the time required for this provides an indication of cell proliferation rate. The nucleoside thymidine is specifically incorporated into DNA during the S-phase and radioactive thymidine has been used in various labelling procedures in order to study the length of the phases of the cell cycle (19). The classic method for the measurement of DNA synthesis time by observing successive waves of labelled mitoses after a single injection of labelled thymidine was inadmissible in our studies. Firstly, the administration of radioactive thymidine to young children whose life expectancy cannot be predicted is unjustified, and secondly, parental permission to obtain serial bone marrow samples is extremely difficult to secure. We have therefore made studies *in vitro*. A double labelling technique was used with ^{3}H-thymidine and ^{14}C-thymidine similar to the double labelling technique described by Hilscher and Maurer (8) and by Wimber and Quastler (20) for studies *in vivo* in which the time of incubation

could be kept short and changes in cell proliferation rate due to the interference with normal physiological conditions diminished. In this technique, administration of ^{3}H thymidine and ^{14}C-thymidine is separated by a known interval and the percentage of cells which become labelled with each compound or with both compounds is determined autoradiographically. Rather than use a single thick emulsion for autoradiography as has been done by several workers (8 14 18 20) we have used a double emulsion technique based on that described by BASERGA and NEMEROFF (2) this allows good staining of bone marrow smears and also unequivocal identification of cells labelled with tritium only. The autoradiographic technique has been described in detail (10)

Materials and Methods

Incubation. Bone marrow aspirates were obtained from either the iliac crest or the sternum in 5 normal subjects and 9 patients with thalassaemia major. The haematological data of these subjects are presented in Table I. After aspiration, the bone marrow was placed in a sterile McCartney bottle containing 18 ml of Heparin-Ringer solution (12). Within one hour the bottle was centrifuged for 10 min at 1500 rpm and the supernatant fluid was discarded. Equal parts of Hanks solution and serum from the same subject were added to the cell suspension in such quantity that the concentration of nucleated cells was about 10,000/mm^{3}. To this suspension, penicillin and streptomycin were added to a final concentration of 0.05 mg/ml. The suspension of bone marrow cells was incubated at 37 °C with ^{3}H-thymidine (specific activity 15 mCi/mM) at a final concentration of 0.5 µCi/ml suspension for one hour. ^{14}C-thymidine (specific activity 0.026 mCi per mM) was then added to a final concentration of 2.5 µCi/ml suspension, and incubation continued for a further ten minutes. The labelling with ^{14}C-thymidine was considered as flash labelling. The cells were then washed in ice-cold 0.9% saline and resuspended in serum. Smears were prepared, fixed 20 min in methanol and washed for 10 min in running water to remove any traces of unbound radioactive material.

Autoradiographs. The bone marrow smears were first coated with liquid emulsion (Gevaert NUC 7 15) by the dipping technique. The emulsion was exposed for one to ten days and then processed. This emulsion registered both ^{3}H and ^{14}C-beta particles. After photographic processing the smears were stained through the emulsion in dilute Giemsa stain at pH 5.75 and the emulsion layer then covered with a layer of celloidin (thickness about 4.5 µm) produced by dipping in a 7 percent solution of celloidin in amyl acetate. A second layer of photographic emulsion (Kodak NTB 2) was then applied by the dipping technique and exposed for 30 to 60 d. The celloidin layer served to preserve the staining of the specimen during the processing of the second emulsion and also to absorb all ^{3}H-beta particles, so that any autoradiographic image in the second emulsion must have been due to ^{14}C-beta particles only (Fig. 1).

Cell counting. CRONKITE *et al.* (6) divided red cell precursors into 5 classes, and we have adopted their classification. The two earliest identifiable stages were designated E_1 and E_2, the more mature elements were termed E_3 (basophilic), E_4 (polychromatic) or E_5 (orthochromatic). In thalassaemic patients, there is a tendency for the developing normoblast to be smaller than normal and it is more difficult to identify the more mature forms (7). Also, very few of the late normoblasts incorporate thymidine (4). The

Table I
Haematological data.

Case No.	Age years	Sex	Hb g/100 ml	Hct	Hb electrophoresis	Erythroid myeloid ratio
			Normal subjects			
1	30	F	14.0	43		
2	16	M	13.8	43		0.3
3	46	F	12.6	39		0.4
4	63	M	13.3	41		0.4
5	63	M	14.6	4		0.3
			Thalassaemic patients (major form)			
6	2	M	3.9	18	A, A ↑ F < 2	5.2
7	9	M	7.0	24	A, A₂↓ F = 10–20*	3.7
8	7	M	6.8	22	A, A₂, F = 15	1.5
9	13	F	7.4	26	A, A₂, F < 10*	1.6
10	14	F	8.0	28	A, A₂, F = 5–10*	2.8
11	5	M	6.5	23	A, A₂↓ F = 50 %	3.0
1	7	M	7.5	26	A, A₂, F ≈ 5	1.6
13	43	M	8.6	29	A, A₂, F = 5–10*	2.2
14	24	F	7.9	27	A, A₂, F ≈ 30–40*	2.5

Patients having low percentage of Hb F had been transfused shortly before the electrophoretic study

more mature normoblasts were therefore omitted in our cell counts and the E_1, E_2 and E_3 (basophilic) normoblasts were considered as single group. Several smears from each subject were examined and, with a few exceptions, at least 1000 red cell precursors (E_1, E_2 and E_3) were examined and classified as being unlabelled, labelled with ^3H-thymidine only or labelled with ^{14}C-thymidine (with or without ^3H-thymidine as well). The number of cells in each group was expressed as percentage of the total number of cells examined.

Calculation of DNA synthesis time and cell cycle time. All cells labelled with ^3H-thymidine must have been in the S-phase during the initial labelling period. During the one hour interval between the first and second labelling number of these cells pass out of the S-phase and will be labelled with ^3H-thymidine only. Cells labelled with ^3H-thymidine which are still in the S-phase when ^{14}C-thymidine is added will also be labelled with ^{14}C-thymidine. Other cells which have entered the S-phase during the one hour interval will also be labelled with ^{14}C-thymidine. The total number of cells labelled with ^{14}C-thymidine represents the number of cells in the S-phase at given instant. As mentioned previously, all ^3H-beta particles are absorbed in the celloidin layer and the second emulsion registers only ^{14}C-beta particles. Thus cells containing only ^3H-thymidine produce no autoradiograph in the second emulsion. It is therefore possible to distinguish cells containing only ^3H-thymidine from cells containing either ^{14}C-thymidine only or both ^{14}C-thymidine and ^3H-thymidine. The duration of DNA synthesis may then be calculated according to the following equation (19)

$$\frac{N_{H}}{N_{^{14}C}} = \frac{T}{T}$$

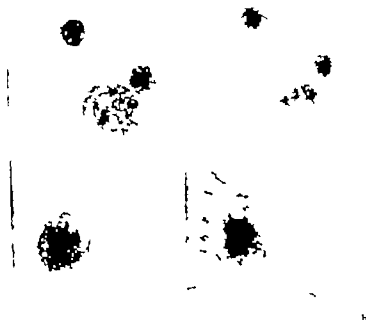


Fig. 1 Double emulsion autoradiograph of erythroblasts in bone marrow smear labelled with ^{3}H-thymidine and ^{14}C-thymidine. *( )* Focus at level of silver grains in first emulsion. Autoradiograph due to ^{3}H-beta particles and ^{14}C-beta particles. *(b)* Focus at level of silver grains in second emulsion. Autoradiograph due to ^{14}C-beta particles only. The lower labelled cell contains ^{3}H-thymidine and ^{14}C-thymidine whereas the upper labelled cell contains ^{3}H-thymidine only

where $N_{^3H}$ = number of cells labelled with ^{3}H-thymidine only
$N_{^{14}C}$ = number of cells labelled with ^{14}C-thymidine,
T — time between first and second labelling,
T = DNA synthesis time.

If T is known and an assumption is made that all cells in the population participate in the proliferative process, then we have also (3, 15)

$$\frac{T}{T} = \frac{N}{N_t} = \frac{N_{^{14}C}}{N_t}$$

where T = cell cycle time
N_s = number of cells in the S-phase
N_t = number of cells the whole population.

Results

Table II gives the data on the percentage labelling of normal and thalassaemic erythroid precursors with H thymidine and

Table II

Labelling of normal and thalassaemic erythroid precursors *in vitro* with ³H-thymidine and ¹⁴C-thymidine.

Case No.	*Haematological* status	*Unlabelled* cells	Cells labelled with ³H-thymidine only	Cells labelled with ¹⁴C-thymidine
1	normal	16.1	4.5	79.4
2	normal	17.9	3.8	78.3
3	normal	18.3	4.4	77.3
4	normal	16.7	4.9	78.4
5	normal	20.2	4.5	75.3
6	thalassaemia major	16.0	6.9	77.1
7	thalassaemia major	17.9	8.4	73.7
8	thalassaemia major	17.[illegible]	7.3	75.3
9	*thalassaemia major*	*17.5*	7.9	74.6
10	thalassaemia major	20.0	8.0	72.0
11	thalassaemia major	19.8	8.8	71.4
12	thalassaemia major	16.4	8.0	75.6
13	thalassaemia major	17.3	6.0	76.7
14	thalassaemia major	15.4	6.9	77.7

Table III

Calculated values for DNA synthesis time and cell cycle time of erythroid precursors *in vitro*.

Case No.	Haematological status	DNA synthesis time in h	Cell cycle time in h
1	normal	17.6	22.1
2	normal	20.6	26.3
3	normal	17.6	22.8
4	normal	16.0	20.4
5	normal	17.6	23.3
		mean 17.9 ± 1.7	mean 23.0 ± 2.3
6	thalassaemia major	11.2	14.3
7	thalassaemia major	8.8	11.9
8	thalassaemia major	10.3	13.6
9	thalassaemia major	9.4	12.6
10	thalassaemia major	9.0	12.3
11	thalassaemia major	8.1	11.3
12	thalassaemia major	9.5	12.8
13	thalassaemia major	12.8	16.7
14	thalassaemia major	11.3	14.3
		mean 10.0 ± 1.5	mean 13.4 ± 1.7

¹⁴C-thymidine. The calculated values of DNA synthesis time and cell cycle time are presented in Table III. The DNA synthesis time was found to be 18 h for normal erythroid precursors and

10 h for thalassaemic cells. The cell cycle times were 23 h and 13 h respectively.

Discussion

A major criticism of the work reported here is that the duration of DNA synthesis as measured *in vitro* may bear little relation to the actual duration of DNA synthesis *in vivo*. However the value of 18 h found for DNA synthesis time in normal erythroid precursors is only slightly longer than the value of 13–14 h found by STRYCKMANS *et al.* (16, 17) using a single injection and observing labelled mitoses *in vivo*. LAJTHA *et al.* (13) using ^{32}P or ^{14}C-adenine *in vitro* found a value of 12–15 hours. By contrast, LALA *et al.* (14) using a double labelling method found that DNA synthesis time *in vitro* was greatly prolonged over the time *in vivo*: their values for dog bone marrow being 17 h and 6 h, respectively. They also found a value of 6 h for DNA synthesis time of human bone marrow *in vivo* which is at variance with the value of 13–14 h reported by STRYCKMANS *et al.* (16, 17).

Our estimate of cell cycle time is not comparable with values found *in vivo* (5, 11) since it is only indirectly derived from the DNA synthesis time, and is based on the assumption that all cells take part in the proliferative process. In spite of possible differences between DNA synthesis times *in vivo* and *in vitro*, the comparison between normal and thalassaemic bone marrow *in vitro* is probably valid. Our results show a distinct shortening of DNA synthesis time and cell cycle time in thalassaemic erythroid precursors. This would indicate an increased rate of proliferation in thalassaemic bone marrow. ASTALDI (1) using colchicine *in vitro* and measuring a stathmokinetic index, has also found a definitely increased proliferative activity in thalassaemic erythroblasts than in normal. Our results suggest that the erythroid hyperplasia characteristic of thalassaemia major is associated with a more rapid turnover of the red cell precursors.

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Summary

Bone marrow cells from normal subjects and thalassaemic patients were incubated for one hour with ^{3}H-thymidine and then ^{14}C-thymidine was added and incubation continued for further 10 min. Smears were prepared for autoradiography and double emulsion technique was followed. The first emulsion registered both ^{3}H- and ^{14}C-beta particles and the second ^{14}C-beta particles only. It was thus possible to distinguish between cells containing H-thymidine only and cells containing both ^{3}H- and ^{14}C-thymidine. From the percentages of labelled cells of each kind the DNA synthesis time and the cell cycle time were calculated. Both were shorter than normal in thalassaemic erythroid precursors.

Zusammenfassung

Knochenmarkzellen von Gesunden und von Patienten mit Thalassämie wurden während einer Stunde mit H Thymidin inkubiert. Dann wurde ^{14}C-Thymidin zugefügt und die Inkubation wurde während weiteren 10 Minuten fortgesetzt. Ausstriche wurden autoradiographisch untersucht, wobei eine Doppelemulsionstechnik angewendet wurde. Die erste Emulsion registrierte H und ^{14}C-Betateilchen, die zweite nur ^{14}C-Betateilchen. So konnte unterschieden werden zwischen Zellen, die nur ^{3}H Thymidin enthielten, und Zellen, die ^{3}H- und ^{14}C-Thymidin enthielten. Aus dem Prozentsatz markierter Zellen beider Art wurden die Zeiten der DNA-Synthese und des Zellzyklus berechnet. Beide waren bei den Erythrozytenvorstufen der Thalassämie kürzer als normal.

Résumé

Des cellules provenant de la moelle osseuse de personnes saines et de malades atteints de thalassémie furent incubées pendant une heure avec de la ^{3}H-thymidine. Après l'adjonction de ^{14}C-thymidine, l'incubation été prolongée pendant encore 10 minutes. Les frottis furent examinés par autoradiographie à l aide d'une technique employant une émulsion double. La première émulsion enregistre les particules bêta de l'^{3}H et du ^{14}C, la deuxième uniquement celles du ^{14}C. De cette façon, les cellules ne contenant que de la ^{3}H-thymidine peuvent être différenciées des cellules contenant aussi bien de la ^{3}H- que de la ^{14}C-thymidine. Le pourcentage des deux espèces de cellules radio-marquées fut employé pour calculer le temps de la synthèse de l'ADN et le temps du cycle cellulaire. Tous deux étaient plus courts q 'ils ne le sont normalement dans les précurseurs érythrocytaires.

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Essentielle Hyperlipämie und verminderte Fibrinolyse Aktivität

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Die Versuche einer ätiologischen Abklärung coronarthrombotischer Komplikationen gehen vielfach von Beobachtungen über Störungen des Fettstoffwechsels und des Gerinnungsmechanismus des Blutes aus. Einen wesentlichen Anteil nimmt dabei die Untersuchung der Fibrinolyseaktivität ein. McDONALD *et al.* (17) sowie EGEBERG (7) fanden bei Patienten mit koronaren «Erkrankungen» eine Erhöhung des Fibrinogenspiegels. HOWELL (13) berichtete über eine Hemmung der Fibrinolyse. Zahlreiche Autoren prüften die Fibrinolyse unter Fettbelastung und kamen zu divergierenden Ergebnissen. GREIG (11), JAMES *et al.* (14), MERSKEY *et al.* (18) sowie HOWELL (13) beobachteten eine Hemmung, NITZBERG *et al.* (19), FEARNLEY (8) und RASMUSSEN *et al.* (22) fanden keinen Einfluß auf die Fibrinolyse.

Zur weiteren Abklärung des Zusammenhanges zwischen gestörtem Fettstoffwechsel und Änderungen der Fibrinolyse haben wir 13 Patienten mit essentieller Hyperlipämie als pathophysiologischen Modellfall einem Kollektiv von Normalpersonen gegenüber gestellt.

Methodik

Zur Bestimmung der Fettwerte (25) erfolgte die Blutentnahme stets morgens etwa 16 Stunden nach der letzten Nahrungsaufnahme. Blut wurde aus der Vena cubitalis gewonnen, in heparinisierten Zentrifugenröhrchen aus Plastik aufgefangen und sofort nach der Abnahme bei 3000 rpm und 4 °C 10 Minuten lang zentrifugiert. Das Plasma wurde abgehoben und unmittelbar darauf nach der Methode von FOLCH *et al.* (9) in der Modifikation von CARLSON (5) extrahiert. Im Extrakt erfolgte die Bestimmung des Lipidphosphors nach BARTLETT (4) (Lipidphosphor × 25 = Phospholipide), des Gesamtcholesterins nach SEARCY *et al.* (26) und der Triglyceride nach CARLSON (3).

Die Bestimmung der freien Fettsäuren erfolgte nach DOLE *et al.* (6).

Die Patienten wurden nach pathologischen Triglyceridwerten ausgewählt (Triglyceridwerte zwischen 214 und 6425 mg%) und standen im Alter zwischen 31 und

64 Jahren. Zum Vergleich dienten 12 Normalpersonen mit Triglyceridwerten zwischen 20 und 164 mg%.

Zur Bestimmung der Fibrinolyseaktivität wurden denselben Versuchspersonen morgens nüchtern 25 ml Blut mit 3,8%igem Natrium citricum tribasicum 1:10 verdünnt in silikonisierten Röhrchen entnommen. Nach Kühlung in Eiswasser wurde das Blut in der Kühlzentrifuge bei 3000 rpm zentrifugiert. Das überstehende Plasma wurde abgesaugt, zum Teil sofort zur Bestimmung der Gesamtfibrinolyse und des Plasminogens verwendet, zum Teil bei −20 °C in silikonisierten Röhrchen zur Bestimmung der Fibrinolyseinhibitoren tiefgefroren.

Die Bestimmung des Fibrins und der Gesamtfibrinolyse erfolgte nach der kjeldahlometrischen Methode von HALSE (12), insofern modifiziert, als zur Erhöhung der Empfindlichkeit 1,0 ml Plasma, 1:15 mit Veronalpuffer verdünnt, statt mit Calciumchlorid mit 0,2 ml einer Thrombinlösung (60 E Topostasin Roche/ml in Veronal-HCl-Puffer pH 7,4) zur Gerinnung gebracht wurde. Für die gesamte Untersuchungsreihe wurde dieselbe Thrombincharge verwendet. Gemessen wurde das Fibrin sofort und nach 24 Stunden Inkubation bei 37 °C im Brutschrank aus demselben Plasma. Die Differenz zwischen beiden Werten wurde als Maß für die erfolgte Fibrinolyse angesehen.

Plasminogenbestimmung

a) *Euglobulin- und Beta-Lipoprotein-Präzipitation.* Die Untersuchung erfolgte nach dem Prinzip von ANDERSSON (1, 2) routinemäßig nach der von GANRY (10) angegebenen Methode: 4,0 ml Citratplasma wurden mit 8,0 ml einer 6×10^{-4} N HCl, die 12,5 E Heparin Vitrum/ml enthielt, gemischt und für 2 Stunden in den Kühlschrank bei +4 °C gestellt. Nachher erfolgte die Zentrifugation bei 3000 rpm durch 10 Minuten, der Überstand wurde abgegossen, das Röhrchen vorsichtig mit Aqua dest. ausgewaschen und das Präzipitat in 4,0 ml Tris-Puffer 0,1 M, pH 7,5, mit Hilfe eines Glasstabes gelöst.

b) *Caseinolyse.* Obige 4,0 ml wurden im Wasserbad auf 37 °C angewärmt und mit 1,0 ml einer Streptokinaselösung (Streptase Behringwerke, 1000 E/ml einer 4% Lösung von Casein Hammarsten in Trispuffer pH 7,5 versetzt. Nach 10 Minuten erfolgte die Zugabe von 3,0 ml einer 4%-Caseinlösung. Eine Minute später wurden 2,0 ml Inkubationslösung als Leerwert entnommen und 2,0 ml einer 10%-v/v Trichloressigsäure (TCA) p. a. hinzugegeben. Weitere 30 Minuten später erfolgte derselbe Vorgang. Beide Proben wurden in den Eisschrank bei +4 °C gestellt, 15 Minuten bei 4000 rpm zentrifugiert und das freigesetzte Tyrosin bei 280 nm in Quarzküvetten mit einem Beckmann DU Spektrophotometer gemessen. Es wurden jeweils Doppelwerte bestimmt. Der Vorteil der angeführten Methode ist der völlig lineare Verlauf der Caseinolyse. Das Ergebnis wurde entsprechend den Empfehlungen der Enzym-Kommission der Internationalen Union für Biochemie (23) in Internationalen Einheiten (I.E.) angegeben, wobei eine Einheit als diejenige Fermentmenge definiert wird, die in einer Minute ein Mikro-Mol Tyrosin aus einem Milliliter Euglobulinlösung unter optimalen Bedingungen freisetzt.

Bestimmung der Fibrinolyseinhibitoren

Sie erfolgten nach der Methode von NORMAN (20). Das dafür verwendete Plasmin wurde aus menschlicher Cohnscher Fraktion III[1] nach der Methode von KLINE (15) hergestellt und mit Streptokinase (Streptase Behringwerke) aktiviert. Die Streptokinase wurde durch mehrmaliges Fällen in 1 mol NaCl-Lösung vom Plasmin getrennt. Sämtliche Inhibitorenbestimmungen wurden in einem Untersuchungsgang und mit derselben Plasminzubereitung durchgeführt und das Ergebnis in Prozenten Hemmung ausgedrückt. Um bei den zu erwartenden höheren Hemmwerten der Hyperlipämiker nicht in den schlecht ablesbaren Anteil der Eichkurve zu kommen – wie sich aus Vorver

[1] Wir danken Dr. M. J. LARRIEU, Centre de Recherches Hématologiques, Paris, für die Überlassung der Fraktion III.

suchen ergab – haben wir jeweils die Hälfte der in den Originalarbeiten angegebenen Plasmamengen verwendet.

) *Bestimmung des «Sofort-Inhibitors»* 0,5 ml Citratplasma wurden mit 2,5 ml Trispuffer pH 7 4 und 4 0 ml einer 4%-Caseinlösung in Trispuffer im Wasserbad bei 37 °C bis zur Anwärmung inkubiert. Dann wurde 1,0 ml Plasminlösung in 0,025 n. HCl hinzugegeben. Die Plasminlösung wurde so eingestellt, daß sich bei halbstündiger Inkubation im inhibitorfreien System eine Extinktionsdifferenz von ca. $\Delta E = 0{,}300$ ergab. Für den 100% Wert wurde Plasma durch Puffer ersetzt. 1 und 31 Minuten nach Plasminzugabe wurden Proben entnommen, in 10% TCA gefällt und das säurelösliche Tyrosin bestimmt.

b) Bestimmung des «Progressiv-Inhibitors». 0,1 ml Citratplasma und 0,9 ml Trispuffer pH 7 4, der 2,2 g Lysin/100 ml zur Verhinderung der Autodigestion enthielt (27) und 1,0 ml Plasminlösung in Trispuffer wurden 180 Minuten bei 20 °C inkubiert und anschließend bei 38 °C einige Minuten vorgewärmt. Dann wurden 1,0 ml Trispuffer und 4,0 ml 4%-Caseinlösung in Trispuffer ebenfalls vorgewärmt, hinzugegeben; 1 und 31 Minuten nach Substratzugabe wurden davon Proben entnommen, mit 10% TCA gefällt und das säurelösliche Tyrosin bestimmt.

Die statistische Auswertung erfolgte mit Hilfe des Student Testes nach den Angaben von Linder (16) Unterschiede von mindestens $p < 0{,}05$ wurden als statistisch signifikant angesehen.

Ergebnisse

Fibrin und Gesamtfibrinolysin

Der Mittelwert des Fibrins betrug für die Normalpersonen $\bar{x} = 276$ mg%, $s = \pm 62$ mg% für die Hyperlipämiker $\bar{x} = 376$ mg%, $s = \pm 24{,}4$ mg% Der Unterschied ist mit $p < 0{,}0005$ bei FG = 31 hochsignifikant. Die absoluten Differenzwerte zwischen dem Fibrin-O- und dem Fibrin 24-Stundenwert als Ausdruck der Fibrinolyse betrugen im Mittel bei den Normalpersonen $\bar{x} = 95$ mg% $s = \pm 54{,}6$ mg% bei den Hyperlipämikern $\bar{x} = 30$ mg% $s = \pm 28{,}9$ mg% Nachdem keine Streuungshomogenität und Normalverteilung vorlag wurde die Irrtumswahrscheinlichkeit nach Van der Waerden und Nievergelt (28) errechnet. Es ergab sich zwischen beidenKollektiven ein hochsignifikanter Unterschied von $p < 0{,}005$.

Plasminogenbestimmung

Gemessen an Tyrosin betrug der Mittelwert für die Normalpersonen $\bar{x} = 355 \cdot 10^{-4}$ I E./ml, $s = \pm 46 \cdot 10^{-4}$ I E./ml, für die Hyperlipämiker $\bar{x} = 348 \cdot 10^{-4}$ I E./ml, $s = \pm 68 \cdot 10^{-4}$ I E./ml. Daraus ergab sich keine signifikante Differenz zwischen den beiden Gruppen.

Fibrinolyseinhibitoren

a) Sofort Inhibitor Der in Prozenten Hemmung ausgedrückte Mittelwert betrug für die Normalpersonen $\bar{x} = 50{,}9\%$ $s = \pm 7{,}9\%$

für die Hyperlipämiker $\bar{x} = 50,6\%$, $s = \pm 9,3\%$. Die Werte für beide Gruppen sind statistisch identisch.

b) Progressivinhibitor. Es ergab sich ein Mittelwert für die Normalpersonen $\bar{x} = 54,4\%$, $s = \pm 6,7\%$, für die Hyperlipämiker $\bar{x} = 63,4\%$, $s = \pm 6,6\%$. Beide Gruppen differieren hochsignifikant mit $p < 0,005$ bei $FG = 24$, $t = 3,6$.

Diskussion

Unsere Untersuchungen ergaben bei Patienten mit essentieller Hyperlipämie gegenüber Normalpersonen, gemessen am Fibrin, eine signifikante Vermehrung des Fibrinogens. Die Gesamtfibrinolyse war bei diesen Patienten ebenfalls signifikant vermindert. Dies könnte auch als eine Erklärung für den erhöhten Fibrinogenspiegel angesehen werden. Die verminderte Fibrinolyseaktivität bei den Hyperlipämikern läßt sich nicht durch eine herabgesetzte Plasminwirkung erklären, da im Vergleich zu den Normalpersonen identische Plasminogenwerte vorlagen.

Es ist naheliegend, die verminderte Fibrinolyseaktivität der Hyperlipämiker mit einer gesteigerten Antiplasminwirkung in Zusammenhang zu bringen. Die Prüfung der Inhibitorenaktivität ergab keine signifikante Vermehrung des «Sofort Inhibitors», jedoch eine deutliche des «Progressivinhibitors». Eine Definition der biochemischen Struktur dieser Inhibitoren steht noch aus, doch soll es sich um Eiweißkörper handeln, die mit Lipiden Verbindungen eingehen können.

Eine zusätzliche Deutungsmöglichkeit der herabgesetzten Fibrinolyseaktivität bieten die elektronenoptischen Untersuchungen von Baló (3), wonach die Anlagerung von Fettpartikeln an Fibrin seine proteolytische Angreifbarkeit hemmen soll. Dagegen spricht jedoch, daß der Zusatz von Triglyceriden *in vitro* keine Änderung der Fibrinolyseaktivität bewirkt.

Die fibrinolytische Aktivität bei Vermehrung einer Fraktion mit Antiplasminwirkung sowie der erhöhte Fibrinogenspiegel bei den Patienten mit essentieller Hyperlipämie können mit großer Wahrscheinlichkeit, zusammen mit der bei diesen Patienten gesteigerten Aggregationsneigung der Thrombozyten (24) als eine der Ursachen für das gehäufte Auftreten coronarthrombotischer Zwischenfälle bei diesen Patienten angesehen werden.

Zusammenfassung

Bei 13 Patienten mit essentieller Hyperlipämie wurden der Fibrinogenspiegel, die Gesamtfibrinolyse, das Plasminogen und die Antiplasmine geprüft. Im Vergleich zu Normalpersonen war das Fibrinogen signifikant erhöht, die Gesamtfibrinolyse deutlich vermindert, das Plasminogen unverändert und von den Antiplasminen das »progressiv wirksame« Antiplasmin ausgeprägt erhöht. Die Bedeutung dieser Veränderungen wird besprochen.

Summary

Fibrinogen level, total fibrinolysis, plasminogen and antiplasmins were determined in 13 patients with essential hyperlipaemia. Fibrinogen was significantly increased in comparison with normal subjects, total fibrinolysis markedly reduced, plasminogen unchanged and, of the antiplasmins, the 'progressively active' antiplasmin was clearly increased. The significance of these changes is discussed.

Résumé

Chez 13 malades atteints d'hyperlipémie essentielle, le taux de fibrinogène, la fibrinolyse totale, l'activité du plasminogène et des anti-plasmines ont été examinés. En comparaison à ceux de personnes normales, le taux de fibrinogène était augmenté de façon significative, la fibrinolyse totale nettement diminuée, l'activité du plasminogène inchangée et, parmi celle des anti-plasmines, l'activité de l'antiplasmine à action progressive augmentée de façon marquée. La signification de ces altérations est discutée.

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Waldenströms Macroglobulinaemia

A Family Study

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Waldenströms macroglobulinaemia (primary macroglobulinaemia) is a rare disorder characterised by a considerable increase in serum globulins of high molecular weight, which are demonstrated by ultracentrifugal analysis. WALDENSTRÖM (21) in 1944 described the clinical syndrome, and he subsequently introduced the term macroglobulinaemia (22) Normally macroglobulins comprise up to 3% of the total serum proteins (4) and a minor increase in serum concentration has been found in amyloidosis, chronic hepatitis, nephrosis, systemic lupus erythematosus, congenital syphilis and occasionally in other diseases (23 15) The clinical and pathological features of primary macroglobulinaemia have been described by several authors (2, 13 14)

We have carried out detailed serological and cytogenetic studies on members of the family of a patient with Waldenströms macroglobulinaemia. These studies are reported and some implications of the findings are discussed.

Case History

Mrs. E. H. aged 62, presented in October 1960 and gave 12 month history of progressive tiredness, dyspnoea, spontaneous bruising, ankle oedema and tendency to recurrent upper respiratory tract and chest infections. She had suffered from rheumatic fever when she was 12 years old and had shingles in 1957 Examination revealed pale, icteric patient with hepato-splenomegaly moderate oedema of the legs, atrial fibrillation and loud, apical pansystolic murmur due to mitral incompetence. Her blood pressure was 190/90. The ocular fundi were abnormal with tortuous, distended veins and occasional haemorrhages and exudates. Her temperature was raised to about 99° F throughout three month stay in hospital.

Laboratory investigations revealed haemoglobin level of 44%, with normal white cell count and platelet count. A blood film showed excessive rouleaux formation.

The blood sedimentation rate was 144 mms fall in one hour (Westergren) the thymol and zinc sulphate turbidities were increased and the total serum protein level was 8 g per 100 ml, with globulin level of 5.5 g/100 ml. The patient was group ARh positive (probably R_1r) and the direct antiglobulin test was positive using an anti-non-gamma globulin reagent. The Sia water test was positive using the criteria outlined by Martin in 1960 (14).

An abnormal densely-stained band was present in the γ region when the patient' serum was subjected to electrophoresis. Immunoelectrophoresis, using technique based on that of Scheidegger (18) with horse anti-human serum (obtained from the Pasteur Institute) revealed prominent band in the γ_1–γ_2 region. Ouchterlony's modification (17) showed that this band was confluent with the band produced by purified preparation of 19S γ_1 globulin. A precipitate was obtained when the patient's serum was added to 0.015 M phosphate buffer pH 7.8 (in the ratio of 1 volume of serum to 10 volumes of buffer) This precipitate gave single band with the above antiserum on immuno-electrophoresis, and similar band was formed when the immuno-electrophoresis was performed using an antiserum known to be specific for anti-γ_1.

Antiglobulin neutralisation tests (10) using an antiglobulin reagent known to contain anti-γ and anti-γ red cells sensitised with anti-D to detect γ globulin, and cells coated with anti-Le^a in the presence of complement (20) to detect γ_1 globulin were carried out. These showed fourfold increase in γ_1 globulin and slight decrease in γ_2 globulin.

Ultracentrifugal studies were kindly done by Mr. R. G. Murray of the Biophysics Department of the Lister Institute. He found an excess of 19S globulins in the patient' serum. It was concluded that the increase of 19S globulins in the patient's serum was due to an increase in the γ globulins.

A specimen of sternal marrow showed an excess of lymphocytes, plasma cells and reticulum cells. The direct Coombs test was positive, the serum bilirubin level varied between 1 and 3 mg/100 ml, and the reticulocyte count was persistently raised. Other positive tests included the Wassermann reaction and the sheep cell agglutination test, but no abnormality was detected in the bleeding and clotting mechanisms, and no cryoglobulins were found. Bence-Jones proteose was not found in the urine and radiological examination of the chest, the skull and the long bones did not show any evidence of multiple myeloma.

Histological examination of percutaneous splenic biopsy specimen showed that 70% of the cells were normal lymphocytes, 17% were polymorphonuclear cells with 5% myelocytes, 4% reticulum cells and the remaining 4% of cells comprised small numbers of premyelocytes, myeloblasts, normoblasts and plasma cells. This appearance was interpreted to indicate extramedullary erythropoiesis. A needle liver biopsy specimen showed little fatty change.

The patient was considered to be suffering from Waldenström macroglobulinaemia, with an associated acquired haemolytic process.

Following her discharge from hospital she rapidly developed an acute haemolytic crisis, with haemoglobin level of 29%. This improved with an increase in the dose of prednisolone and blood transfusion, but she developed diabetes mellitus, which was presumed to be caused by the cortico-steroids. Blood sugar levels were readily controlled on oral tolbutamide.

For the subsequent two years Mrs. E. H., was in remission and, although she still complained of dyspnoea on moderate exertion, was able to lead relatively normal life. Her haemoglobin levels varied between 70% and 100%, but the blood sedimentation rate was persistently raised to above 70 mm fall in one hour (Westergren) and the Sia Test was always positive.

A steady deterioration in her clinical condition started in August, 1963 when she again developed severe dyspnoea. Clinical examination showed excessive bruising,

Table I
Pathological data in the relatives.

Case	Hb. g%	Wbc. per mm³	E.S.R. mm/h	Latex agglut. tests R. A.	Latex agglut. tests L. E.	Blood group	Total serum protein g%
C.J	12.6	4 400	38	-ve	-ve	A.Rh. +ve.	7 7
A.L.J	13.4	3,200	6	-ve	-ve	A.Rh. +ve.	6 9
M.B.	13.0	4,200	2	-ve	-ve	A.Rh. +ve.	6.8
A.C.J	13.4	6,800	1	ve	ve	A.Rh. +ve.	6.5
E.W	12 7	5,400	2	-ve	-ve	0.Rh. +ve.	6.2
G.W J	11 7	6,200	3	-ve	-ve	A.Rh. +ve.	6.8

hepatosplenomegaly peripheral oedema, atrial fibrillation and mitral incompetence with considerable cardiac enlargement. Her haemoglobin level was 49%, but there was no evidence of haemolysis and the Coombs Test was negative. The Sia test was positive. She was treated with mersalyl, digitalis, prednisolone and blood transfusion, and terminally a course of chlorambucil was tried without obvious clinical benefit.

Post mortem examination showed the typical, diffuse, pleomorphic infiltrate of the marrow and lymph nodes (11) N evidence of malignancy was found (3).

Family Study

A full medical history was taken from the relatives and careful examination was conducted in each case (Fig. 1) Brief clinical details are described and the pathological findings are listed in T ble I.

Sister of Mrs. E. H Mrs. C. H., aged 58, gave history of 'encephalitis' in 1961. This illness was characterised by persistent pyrexia, confusion, convulsion and prolonged stupor An electroencephalogram showed diffuse changes consistent with encephalitis, and examination of specimens of cerebrospinal fluid revealed raised protein levels of up to 108 mg/100 ml, with increase levels and white cell counts of about 70 lymphocytes. The total serum pro t that time was 5.9 g/100 ml, with serum albumin level of 3.5 g/100 ml globulin level of 2.4 g/100 ml: the blood
imentation rate was 28 mm hour (Westergren) Ultracentrifuge and
mosome studies were not pe g this illness. Neurological examination
on later revealed mild de other abnorm physical signs. Clinical
tion of the three child did not y major disorder
M. D and A. L. J)

Table I (continued)

Serum alb. g%	Serum glob. g%	Sia test	Paper electro-phoresis	W R.	Immuno-electro-phoresis	Antiglob. neutral. test
3.7	4.0	-ve	inc'd band γ-region	-ve	Prominent γ_1-glob. band.	γ -glob. inc'd
3.9	5.0	-ve	normal	-ve	normal	normal
4.0	2.8	-ve	normal	-ve	normal	normal
3.8	2.7	-ve	normal	-ve	normal	normal
3.6	2.6	-ve	normal	-ve	normal	normal
3.6	3.2	-ve	slightly inc'd band γ-region	-ve	normal	normal

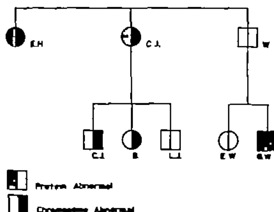


Fig. 1

Brother of Mrs. E. H. Mr W J aged 64 was admitted into hospital in August, 1963 with myocardial infarction. H developed pneumonia, which was shown to be caused by penicillin-resistant staphylococcus and he subsequently died in November 1963 from congestive heart failure. Paper electrophoresis of the serum showed considerable rise in gamma globulin which was ttributed to the pulmonary infection. The latex fixation tests for rheumatoid arthritis and systemic lupus erythematosus were

negative, as were the Sia water test and the Wassermann reaction. Further serological and chromosome studies could not be performed. Neither of his children (G W J. and E. W) gave significant histories and were normal on examination.

Cytogenetic Data

Chromosome studies were carried out on all members of the family except W J., using the peripheral blood culture method of Hungerford *et al.* (7) The results of the chromosome counts are shown in Table II.

The propositus and two of the relatives were shown to have an abnormal cell line with an abnormal large chromosome in addition to the normal diploid complement, the extra autosome being similar to that found in other cases of primary macroglobulinaemia (3) E. H. had two cells (4%) with 47 chromosomes the abnormal chromosome was metacentric (type I) in one cell and submetacentric (type II) in the second cell. M. B. had one typical macroglobulinaemia cell (1.33%) with a submetacentric chromosome (type II) and G. W J had a similar abnormal cell (2%) containing submetacentric chromosome (type II)

In addition to these aneuploid cells, pseudo-diploid cells which contained a large submetacentric chromosome (type II) were found in four of the seven cases which were investigated. Particular care was taken to ensure that groups 1 3 contained full complement of chromosomes.

E. H One pseudo-diploid cell (2%) was found with a number 21–22 chromosome missing

C J Two pseudo-diploid cells were present (8%). In one of these cells a number 19–20 autosome was missing, and in the second cell a member of the group 6–12 was absent.

A. C. J Two pseudo-diploid cells were found in which a member of the group 6–12 was missing (2%)

C. W J Large submetacentric chromosomes (type II) were found in two pseudo-diploid cells (4%) In one of these cells number 17–18 autosome was missing and in the other cell a small member of the group 6–12 was absent.

These pseudo-diploid cells may have suffered loss of the small chromosome during the preparation of the chromosomes for study and these cells may be 'macroglobulin' cells rather than additional stem cell lines. A fuller discussion of the incidence and significance of the pseudo-diploidy will be presented in separate communication.

Cells containing abnormal chromosomes were found, therefore, in five members of the family Two other relatives were investigated, but no abnormal cell lines were discovered. The results are summarised in Table III.

Discussion

A rise in the level of γ_1 globulin in the serum has been demonstrated in one of the relatives of our patient with primary macroglobulinaemia, and serum electrophoresis suggested a rise of serum globulin in another relative, although this was not confirmed by globulin titration. In the case of C. J the rise of serum globulin levels followed an attack of encephalitis two years previously

At least two instances of familial protein abnormalities have been reported in macroglobulinaemia. Massari *et al.* (16) in-

Table II
Results of the chromosome counts in the family

Case	Percent distribution							No. of cells counted
	<44	44	45	46	47	48	>48	
E. H.	6	6	8	76	4	0	0	50
C. J	4	8	4	84	0	0	0	25
A. L. J	4	8	8	80	0	0	0	25
M. B.	4	4	4	84	4	0	0	25
A. C. J	8	0	8	84	0	0	0	25
E. W	4	4	8	80	0	4	0	25
C. W J	8	0	12	76	4	0	0	25

Table III
The incidence of abnormal cells in this family

Case	No. of abnormal cells	No. of cells counted	% of abnormal cells	Abnormal 47M	Cells 46M
E. H.	3	50	6	2	1
C. J	2	25	8	0	2
A. L. J	0	50	0	0	0
M. B.	1	75	1.33	1	0
A. C. J	2	100	2	0	2
E. W	0	100	0	0	0
C. W J	3	50	6	1	2

vestigated two brothers suffering from Waldenströms syndrome and reported that the mother had an increase in gamma globulin on paper electrophoresis of the serum, and an increase in beta 2 globulin has been demonstrated in a mother and son by SELIGMANN and BADEN (19) Another family study failed to reveal any abnormalities in the relatives of a patient with primary macroglobulinaemia by estimation of the serum protein levels, paper electrophoresis and the Sia water test (9)

Chromosome abnormalities have been found in a number of patients with Waldenströms macroglobulinaemia (2, 6, 1 5) The abnormal chromosome may be either metacentric (type I) or submetacentric (type II) Similar abnormal chromosomes have also been observed in lymphoid cells from individuals with mild degrees of gamma-globulinaemia (5) The family described above is, we believe, the first in which the abnormal chromosome has been found

to have a familial incidence. Five of the seven members of the family studied possess lymphoid cells containing an abnormal large chromosome. In all cases the chromosome present was of type II morphology although one type I cell was seen in the propositus. The presence of the abnormal genetic material was associated in only one case with macroglobulinaemia. The other affected individuals had only slight elevation of the γ globulin level or none at all. This finding confirms the finding in a previous paper (5) that the chromosome is not always accompanied by macroglobulin over production.

The small proportion of lymphocytes which demonstrate a chromosome abnormality suggests that they may originate in the later stages of embryonic or post natal development and it may be suggested that they may be the source of the abnormal serum proteins.

The incidence of the abnormal cells in this family would strongly suggest that their production is genetically determined by an autosomal gene or genes. A situation which has not been reported in man.

It is evident that the chromosome aberration precedes the protein abnormality and may lie dormant for some years. It has been suggested that an antigenic stimulus may provoke abnormal protein production (5). It is relevant in this context to refer again to the patient Mrs. C. H., who suffered from encephalitis in 1961. This illness resembles the encephalopathies described in patients with Waldenströms macroglobulinaemia (12) but at that time she had normal serum protein levels. Two years later she had a raised serum globulin level, and chromosome aberrations affecting 8% of lymphocytes have been detected. This case is evidence that these abnormal cells can be stimulated to abnormal protein production by an antigenic reaction.

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Summary

A case of Waldenströms macroglobulinaemia has been described and biochemical, serological and cytogenetic studies have been performed on this patient and six of the members of the family. Chromosome abnormalities were found in four relatives, one of whom showed a rise in serum γ_1-globulin. The genesis of the chromosome aberrations and the serum protein abnormalities have been discussed.

Zusammenfassung

Es wird ein Fall von Makroglobulinämie Waldenström beschrieben. Bei diesem Patienten und bei sechs Angehörigen seiner Familie wurden serologische und zytogenetische Untersuchungen vorgenommen. Chromosomenanomalien fanden sich bei vier Verwandten, von denen einer eine Zunahme der γ_1-Globuline im Serum aufwies. Die Genese der Chromosomenveränderungen und die Anomalien der Serumproteine werden diskutiert.

Résumé

Un cas de macroglobulinémie de Waldenström est décrit. Des examens sérologiques et cytogénétiques ont été faits chez ce malade et chez 6 membres de sa famille. Des anomalies chromosomiales furent mises en évidence chez 4 parents dont un présentait une augmentation des gamma-globulines. La pathogénie des altérations chromosomiales et des anomalies des protéines sériques est discutée.

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Proliferative Activity of the Cells of Acute Leukaemia in Relapse and in Steady State*

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It has been well known since 1959 that, by studying the labelling index (L.I.) with tritiated thymidine, the proliferative activity of acute leukaemia cells is constantly less than that of normal bone marrow blasts (1—3) but varies greatly from one case to another (4—9). This variation led us to study whether there is any relationship between the proliferation rate of blast cells and the stage of evolution of the disease. In fact, even without any treatment, the blast population may be either expanding spontaneously or maintaining a steady state. A study of the different evolution stages of the disease in relation to treatment has just been published by Schmid *et al.* (10—11).

In the present research the proliferative behaviour of expanding and steady state blast population was investigated in untreated acute leukaemia patients. For this purpose, we made an *in vitro* study of thymidine H^3 incorporation in the blasts of 24 acute leukaemia patients in steady state and compared the results with those obtained in a second group of 20 patients with expanding blast population. Furthermore, according to the observation of a previous investigation (12) that, within the same acute leukaemia population, the proliferative activity in the blast cells of various sizes is different, we tried to establish the proliferative behaviour of the various blast classes in the different growth conditions.

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Table I
Labelling indices of acute leukaemia blast cells.

Evolutive condition					
Steady			Expanding		
Cases	Bone marrow	Peripheral blood	Cases	Bone marrow	Peripheral blood
1	7.3	—	1	20.0	—
2	1.5	—	2	12.5	—
3	9.1	—	3	12.0	3.8
4	4.4	—	4	—	8.8
5	9.3	—	5	13.2	8.2
6	9.8	—	6	8.8	1.3
7	7.2	3.2	7	8.4	—
8	13.7	5.5	8	16.7	11.6
9	11.0	—	9	19.0	6.7
10	10.6	—	10	14.4	0.9
11	5.9	2.5	11	30.0	3.2
12	6.4	—	12	8.4	9.0
13	6.0	—	13	12.0	5.0
14	11.9	0.9	14	13.3	12.9
15	2.8	—	15	21.3	16.4
16	8.3	—	16	4.7	1.0
17	7.3	—	17	—	17.2
18	5.8	—	18	15.0	—
19	5.3	0.9	19	10.0	—
20	8.5	0.4	20	23.4	16.5
21	3.2	0.6			
22	13.0	3.3			
23	3.3	1.8			
24	10.5	1.6			
Mean value	7.59	2.20		14.67	8.31

Variance analysis of the labelling indices in the two evolutive conditions
for peripheral blood $F = 9.71$ $(F_{0.01} = 4.30)$
for bone marrow $F = 22.90^*$ $(F_{0.01} = 7.35)$

Materials and Method

44 cases of acute leukaemia were studied in all. The subjects were adults and had never previously been studied. Two groups of acute leukaemia were distinguished. (a) forms with steady state blast population (24 cases) (b) forms with blast population expanding (20 cases)

I 23 of the 44 cases, the study was carried out on bone marrow material and peripheral blood at the same time. In preparing the smears of cell suspensions, tried to chieve equal cell concentration per unit of surface. Cell diameters were measured and the blast population of each case considered was divided into 3 cell classes (large, medium, small blasts) A control was made to observe whether for the same case, cell

diameter was significantly influenced by belonging to different smears. Measurements taken on different smears with constant cell concentration per unit of surface did not reveal significant differences (χ^2 not significant)

After aspiration with heparinized syringe, the marrow and venous blood was diluted / or / with TC 199 and left to incubate for 1 h in rotating system at 37 °C in the presence of 2—5 μc/ml of thymidine-H^3 (specific activity 14.5 c/mM) The smears were fixed in Carnoy and covered with nuclear emulsions (Ilford L_4 or K_2) which had previously been diluted / with distilled water and left exposed for 3—10 days at 4 °C. After developing and fixing, the smears were coloured with May-Grunwald Giemsa buffered at pH 7 4

Results

Table I show the labelling index with thymidine-H^3 of the bone marrow blast cells in the two evolution conditions. In the acute leukaemias in steady state the L.I turned out to be signif icantly lower than those of the acute leukaemias in expanding phase both in marrow and peripheral blood. In the acute leukaemias in both state and in relapse the L.I. of the whole population was significantly higher in the bone marrow than in the peripheral blood

In cases where three different sizes of blasts were considered and percentage distribution evaluated for each single case, we observed a clear dishomogeneity in the proportion of cells of different diameter between marrow and peripheral blood and this was always relatable to a greater percentage of large cells in the marrow blood (Table II) In expanding forms we also observed a higher percentage of cells with larger diameter as compared with steady state forms both at marrow and peripheral blood (Fig 1)

Studying the L.I s for each single class, we constantly observed that in classes of large diameter the L.I s are distinctly higher than those of the smaller classes. More specifically in some marrow blast cells of large diameter the L.I. reaches values on a par with those of the blast cells in normal marrow (Fig 2) No statistically significant differences were observed in the L.I. s of the large cell classes between the steady state and expanding groups of leukaemias either in the marrow or circulating blood.

Discussion

The proliferative activity as expressed by the L.I., was significantly higher in cases where the blast population was ex panding This proved to be true both for bone marrow and cir

Table II
Percent of blasts with different diameter in bone marrow and peripheral blood.

Cases	Bone marrow Small cells	Medium cells	Large cells	Cases	Peripheral blood Small cells	Medium cells	Large cells
1	28.0	57.0	14.9	1	31.3	56.5	12.1
2	37.0	52.9	9.9	2	36.0	53.3	10.9
3	22.1	63.6	14.2	3	28.7	63.6	5.5
4	34.9	56.3	8.7	4	44.2	49.5	6.1
5	18.5	58.7	22.7	5	48.9	48.6	2.5
6	17.5	64.3	18.8	6	40.8	50.7	8.4
7	25.3	60.3	14.3	7	16.0	69.3	14.6
8	23.4	63.1	13.4	8	34.7	57.2	7.9
9	21.3	72.0	6.6	9	30.1	64.5	5.5
10	27.3	60.9	11.7	10	23.5	65.2	11.2
11	20.9	68.6	10.3	11	21.9	75.1	2.8
12	37.3	58.3	4.2	12	34.3	61.4	4.2
Mean value	27.77	60.86	11.24		32.53	60.12	7.23

t not significant for small and medium cells,
t significant for large cells of bone marrow and peripheral blood ($t = 2.31$ $t_{0.05} = 2.20$)

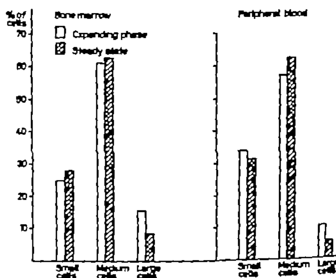


Fig. 1 Percent of blasts with different diameter in the two evolutive conditions (bone marrow and peripheral blood)

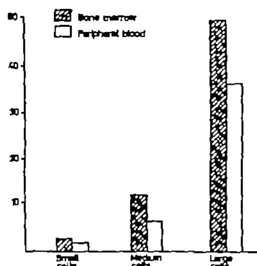


Fig. 2. Labelling index in the different blast classes of bone marrow and peripheral blood (mean value of *11* cases in relapse).

culating blast cells. The greater proliferative activity of the blast cells as observed in these clinical forms fully agrees with the hypothesis that neoformed blasts retain normal or almost normal proliferative activity and that this activity steadily decreases as the cells grow old (12)

As demonstrated by previous *in vivo* research, large blast cells change into small and not proliferating blasts in the course of successive cell divisions (12 13) The large cells of acute leukaemia divide at a rhytm very similar to that observed in blast cells of normal bone marrow (14) The fall in the L. I. on the progressive reduction in cell diameters might depend on the accumulation of non proliferating blasts (1? 13 15)

All these facts suggest that the degree of transformation of the larger blasts in the smaller ones might in itself constitute a regulator factor of the rhythm of expansion of the blast population. Particularly in the cases of acute leukaemias in relapse phase, the expansion of the leukaemic population might be a consequence of the fact, that after division, a higher number of large cells retain their parental characteristics, namely they remain large after division On the other hand, since the L. I. s of the largest blast cells are not significantly different in the two evolutive conditions, the expansion of the blast population cannot be related to a different proliferative

capacity on the part of these highly proliferating blasts. This possibility that a proliferating and non proliferating population coexist in human acute leukaemias is in agreement with what has already been observed by some authors in various tumors (16—21)

Summary

The labelling index (L. I.) of bone marrow and peripheral blast cells was determined with thymidine-H^3 in expansion and steady state conditions of the leukaemic blast population. A higher L. I. in the total population was observed in the expanding forms and these forms also presented a higher percentage of large and more actively proliferating blasts both in bone marrow and peripheral blood. In all cases the bone marrow L. I. was higher than that in the peripheral blood. A higher percentage of large cells was observed in the bone marrow. The transformation of large blasts into small is considered as a possible regulator mechanism of the expansion of the blast population and responsible for the accumulation of blasts in a non-proliferating compartment.

Zusammenfassung

An leukämischen Blasten-Populationen im Stadium der Ausbreitung und bei stationärem Verhalten wurde mit H^3 Thymidin der Markierungsindex von Knochenmarks- und Blutzellen bestimmt. Bei Formen im Stadium der Ausbreitung fand sich ein höherer Markierungsindex der gesamten Population. Diese Formen zeigten ferner einen höheren Prozentsatz grosser und aktiver proliferierender Blasten in Knochenmark und peripherem Blut. Bei allen Fällen war der Markierungsindex im Knochenmark höher als im peripheren Blut. Im Knochenmark fand sich ein höherer Prozentsatz grosser Zellen. Es wird angenommen, daß die Umwandlung grosser in kleine Blasten einen Regulationsmechanismus der Ausbreitung einer Blasten-Population darstellt und für die Ansammlung von Blasten in einem nicht proliferierenden Bezirk verantwortlich ist.

Résumé

L'index de marquage de la moelle osseuse et des blastes périphériques a été déterminé à l'aide de thymidine tritiée dans une population de blastes leucémiques au stade d'expansion et au stade stationnaire. Un index plus élevé a été trouvé dans les formes en état d'expansion, ces formes présentant un pourcentage plus élevé de grands blastes proliférant plus rapidement aussi bien dans la moelle osseuse que dans le sang périphérique. Dans tous les cas, l'index de marquage était plus élevé dans la moelle osseuse que dans le sang périphérique. Un pourcentage plus élevé de grands blastes a été trouvé dans la moelle osseuse. La transformation de grands en petits blastes est considérée comme étant un mécanisme possible de régulation de l'expansion de la population des blastes, mécanisme qui serait aussi responsable de l'accumulation de blastes dans un compartiment sans prolifération.

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In vitro Synthesis of Haemoglobin from Fe^{59} and Leucine-C^{14} by Normal, Sickle-Cell and Thalassaemic Immature Red Cells

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and P. Christakopoulos

Haemoglobin biosynthesis has been studied extensively either *in vivo* or *in vitro*. Chromatography and electrophoresis contributed greatly to the separation of the various normal and abnormal haemoglobins and also to the fractionation of the soluble portion of the red cells.

Numerous investigators have shown that iron and aminoacids enter the immature red cell and are incorporated into haemoglobin. It is further known that both iron and aminoacids, after entering the immature red cells, form intermediate products, before the final formation of haemoglobin (10, 18, 22, 23). It seems that the process of synthesis of haem and globin may proceed independently (4, 7, 11). Radioactive iron (Fe^{59}) can serve to follow the synthesis of haem, and leucine-C^{14} the synthesis of the globin part (9, 19).

It is known that the most prominent biochemical abnormality in thalassaemia is a quantitative decrease in the synthesis of Hb A. Marks and Burka (16) suggest that in thalassaemic reticulocytes there is a decrease in beta chain synthesis, and secondary of the alpha. Bank and Marks (3) also have shown that the endogenous capacity of ribosomes prepared from cells of thalassaemic subjects, to incorporate aminoacids is markedly diminished compared to that of non thalassaemic subjects. Karpatkin (12) also has reported a defect in globin synthesis. Bannerman *et al.* (4, 5) suggested that there is a quantitative impairment of haemoglobin synthesis by thalassaemic immature red cells. They thought that this was due to an impairment of haem synthesis as there was no evidence of any

alteration in the globin synthesis. VAVRA *et al.* (21) have shown two abnormalities in thalassaemic immature red cells (1) a defect of glycine utilization to form aminolevulinic acid and (2) a defective step following the formation of protoporphyrin or haem. STEINER *et al.* (20) have shown an enzymatic defect of haem synthesis in thalassaemia.

The present work deals with the fractionation, employing gel filtration, of the soluble portion of the red cells from normal subjects, sickle-cell patients and patients with thalassaemia, after incubation with Fe^{59} or leucine-C^{14} or both simultaneously

Materials and Methods

The study has been performed in blood from 36 normal subjects* 35 thalassaemic patients and 7 sickle-cell anaemia patients. Blood samples were collected by venipuncture in heparinized syringes. A suspension of blood was prepared by mixing 3.2 ml of blood and 0.8 ml of glucose-saline (10 mg/ml in 0.9% saline). In the above suspension 3 μc of Fe^{59} in citrate buffer or 5 μc of L-leucine-C^{14} or both simultaneously were added. Incubation were carried out in an O_2 atmosphere at 37 °C in water bath. Blood samples from 20 normal subjects and 20 thalassaemic patients have been incubated with Fe^{59} for 6 h. Of the remaining samples (7 normal, 5 thalassaemic and 5 sickle-cell anaemia samples) have been incubated simultaneously with Fe^{59} and leucine-C^{14} for 2 h and the rest (9 normal, 10 thalassaemic and 2 sickle-cell anaemia) with leucine-C^{14} only for 2 h.

The incubation was followed by 9 washings of the red cells with isotonic saline and the cells were then haemolyzed (1 ml red cells in 9 volumes distilled water) The stroma was removed by centrifugation at 40,000 g for half an hour The haemolyzates were passed through cation exchange CM-Sephadex C-50 column and fractions of 5 ml were collected. Details of the chromatographic processing are described by MALAMOS *et al.* (14)

The radioactivity of the collected samples, containing only Fe^{59} was measured in well-type scintillation counter (Nuclear Chicago, Model 132) and of the samples containing both Fe^{59} and leucine-C^{14} in liquid scintillation spectrometer (TRI-CARB) Packard. For the samples which were measured in the TRI-CARB, the quenching due to colour and protein content was corrected by the method of internal standardization.

Spectrophotometric determinations of the absorbancy of the samples were made with Zeiss PMQ II spectrophotometer

Results

(a) For Fe^{59}

After chromatography radioactivity appears in two clearly separated peaks in all cases studied i. e normal subjects, thalassaemic patients and sickle-cell disease. The recovery of the added

* Refering to normals, patients with blood loss anaemia, were examined for this study This as they have elevated numbers of reticulocytes whereas completely normal persons would not have them.

counts was 92 %. First radioactive peak showed a maximum absorbancy at 280 nm. The second peak appears together with haemoglobin and shows a maximum absorbancy at 410 nm.

Normal samples. The ratio of the first to the second peak in 7 samples, after 2 h incubation, was 0.25±0.10. The same ratio in 20 normal samples, after 6 h incubation was 0.47±0.04. Thus the radioactivity of the second peak, which consists of haemoglobin, is significantly higher (Fig. 1).

Thalassaemic samples. The radioactivity of the first peak was much higher than the radioactivity of the second peak which consisted of haemoglobin. The ratio of the first to the second peak in 5 thalassaemic samples after 2 h incubation was 6.47±3.47. The same ratio in 20 thalassaemic samples after 6 h incubation, with Fe^{59} only was 2.42±0.33 (Fig. 2).

Sickle-cell disease patients. After 2 h incubation with Fe^{59} of 5 samples the ratio of the first to the second peak was 0.15±0.04 (Fig. 3).

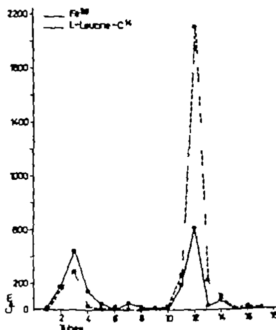


Fig. 1. Representative curve. Normal subject, simultaneous (2 h) incubation with leucine-C^{14} and Fe^{59}.

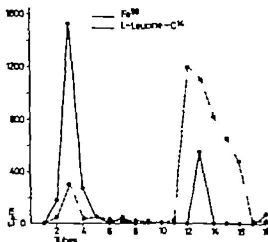


Fig. 2. Representative curve. Cooley patient, simultaneous (2 h) incubation with Fe^{59} and leucine-C^{14}

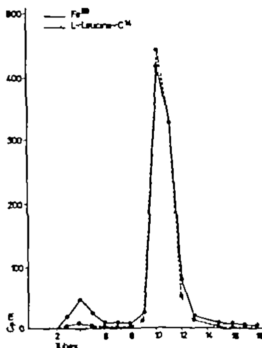


Fig. 3. Representative curve from sickle-cell anaemia patient, simultaneous (2 h) incubation with Fe^{59} and leucine-C^{14}

Table I

Ratio of the first to the second radioactive peak (Mean value ± error of the mean value).

Substrate	Normal subjects	Sickle-cell disease patients	Thalassaemic patients
		2 h incubation	
Fe^{59}	0.25 ± 0.10	0.15 ± 0.04	6.47 ± 3.47
Leucine-C^{14}	0.27 ± 0.08	0.12 ± 0.02	0.25 ± 0.05
		6 h incubation	
Fe^{59}	0.47 ± 0.04		2.42 ± 0.33

(b) For Leucine C^{14}

The chromatographic pattern of the samples, revealed two radioactive peaks in the same position with those described for Fe^{59}. The recovery of the added counts was 80–85%. The ratio of the radioactivity of the first to the second peak was in 16 normal 0.27 ± 0.08, in 15 thalassaemic samples 0.25 ± 0.05 and in 8 sickle-cell disease patients 0.12 ± 0.02.

Discussion

In the present study the haemolysates of red cells (normal, sickle-cell, thalassaemic) give chromatographically two peaks one non haemoglobin and one haemoglobin both of which can be labelled with Fe^{59} or leucine-C^{14}.

For the radioactivity due to Fe^{59} after 2 h incubation, the first peak (non haemoglobin) is lower than the second peak (haemoglobin) in normal (ratio 0.25 ± 0.10) and in sickle-cell anaemia (ratio 0.15 ± 0.04) and in thalassaemia the first (non-haemoglobin) peak is higher than the haemoglobin (ratio 6.47 ± 3.47).

For the radioactivity due to leucine-C^{14} after 2 h incubation the non-haemoglobin peak is lower than the haemoglobin for all the cases studied: normals (ratio 0.27 ± 0.08), sickle-cell (ratio 0.12 ± 0.02) and thalassaemia (ratio 0.25 ± 0.05).

As has been reported previously the first peak of radioactivity after 2 h incubation, either with Fe^{59} or leucine-C^{14} is a non-haemoglobin fraction. The existence of a non-haemoglobin fraction in the soluble portion of the red cells has been described by numerous investigators in man and in animals. Thus WIGGANS *et al.* (22) have shown the existence of such a fraction in the nucleated erythrocytes of ducks.

RABINOVITZ and OLSON (18) studying the aminoacid incorporation into the protein fraction of rabbit reticulocytes, suggest that this iron containing protein fraction is a precursor to haemoglobin. The existence of a non-haemoglobin iron containing fraction in the soluble portion of the red cells has been indicated also by ZAIL *et al.* (23) ALLEN and JANDL (2) and NOYES *et al* (17) after incubation of peripheral blood from man or animals with Fe^{59} GREENOUGH *et al.* (10) studied this fraction in bone marrow of man and dogs after incubation with Fe^{59} and leucine-C^{14} simultaneously FALBE HANSEN and LOTHE (8) studied this fraction in blood of men under normal and pathological conditions and in animals, after incubation with Fe^{59} Such a fraction with similar properties, has been described by MALAMOS *et al.* (15) in thalassaemic patients after incubation of blood with Fe^{59} and chromatography of the haemolyzates.

The non-haemoglobin containing fraction is considered by all, who have studied it, as related to haem synthesis, but its nature is under discussion. RABINOVITZ and OLSON (18) suggest that a protein fraction associated with reticulocytes ribonucleoprotein is a precursor to haemoglobin. According to MALAMOS *et al* (15) the spectrum of the non-haemoglobin iron containing fraction, its high molecular weight and acidic nature, suggest a nucleic acid. ZAIL *et al.* (23) showed that after bone marrow incubation with iron, iron appeared in a fraction isolated on column chromatography which consisted of ferritin and an active haemoglobin precursor Finally it has been shown that it consists of more than one fraction among which one is ferritin (13)

The present work indicates the presence of a non haemoglobin fraction containing Fe^{59} and leucine C^{14} The interesting finding of this study is that in thalassaemic samples, the ratio of the first to the second radioactive peak for Fe^{59} is above unity and for leucine C^{14} below unity

After 6 h incubation of thalassaemic samples with Fe^{59} the non-haemoglobin radioactive peak still remains higher than the haemoglobin peak, but the ratio is decreased in comparison to the results after 2 h incubation, because of slight increase in the radioactivity of the haemoglobin peak. This occurs probably because iron of the first peak is probably slowly released for haemoglobin synthesis.

The present study is not a quantitative one and the high radioactivity of the first peak due to Fe^{59} in contrast to the low observed

for leucine-C^{14} do not give any information for defect in haem synthesis or for a normal globin synthesis, although for both haem (4, 21) and globin synthesis (12, 16) defects have been described.

The high radioactivity of the first peak for Fe^{59} perhaps is due to a higher iron pool, a suggestion which has been made by BANNERMAN *et al.* (4) and also large amounts of iron, in the form of ferritin and haemosiderin have been revealed in thalassaemic erythropoietic cells by electron-optical techniques (6). As for the pattern of the radioactive peaks due to leucine-C^{14} which resembles normal a probable explanation is a lower leucine pool known that immature red cells contain among their free aminoacids leucine (1).

The study is further continued in order to elucidate quantitative disturbances.

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Summary

Blood samples from normal subjects, thalassaemic and sickle-cell anaemia patients were incubated with Fe^{59} and leucine-C^{14}. The distribution of Fe^{59} and leucine-C^{14} in the soluble portion of the erythrocytes was studied, by means of gel cation exchange chromatography. After chromatography of all the samples studied, the radioactivity appeared in two clearly separated peaks, the first non haemoglobin and the second consisted of haemoglobin. The ratio of the Fe^{59} radioactivity of the first to the second peak was below unity in normal and sickle-cell anaemic haemolysates and above unity in thalassaemic haemolysates. Contrary the ratio of the leucine-C^{14} radioactivity was below unity in all the samples studied, even in the thalassaemic ones. The interpretation of the results is discussed.

Zusammenfassung

Blutproben von Gesunden sowie von Patienten mit Thalassämie und mit Sichelzellanämie wurden mit Fe^{59} und Leucin-C^{14} inkubiert. Die Verteilung von Fe^{59} und Leucin-C^{14} im löslichen Anteil der Erythrozyten wurde mit Hilfe der Gelkationenaustauscher-Chromatographie untersucht. Nach der Chromatographie zeigte die Radioaktivität in allen untersuchten Proben zwei scharf getrennte Gipfel, von denen der erste kein *Hämoglobin* aufwies, während der zweite aus *Hämoglobin* bestand. Das Verhältnis der Fe^{59}-Radioaktivitäten im ersten und zweiten Gipfel lag bei Hämolysaten von Gesunden und von Patienten mit Sichelzellanämie unter 1, bei Hämolysaten von Thalassämikern betrug es mehr als 1. Im Gegensatz dazu war das Verhältnis der Leucin-C^{14}-Radioaktivitäten bei allen Proben, auch bei denjenigen von Thalassämikern, kleiner als 1. Die Interpretation dieser Ergebnisse wird diskutiert.

Résumé

Des échantillons de sang venant de sujets normaux et de malades atteints de thalassémie et de drépanocytose ont été incubés avec du Fe^{59} et de la leucine-C^{14}. La distribution du Fe^{59} et de la leucine-C^{14} dans la partie soluble des érythrocytes a été étudiée à l'aide de la chromatographie sur gel échangeur de cations. La chromatographie de tous les échantillons mit en évidence deux pics distincts de radio-activité, le premier ne contenant pas d'hémoglobine, le second en contenant. La relation des taux d'activité du Fe^{59} entre le premier et le second pic était en dessous de un dans les hémolysats normaux et dans ceux provenant d'anémies falciformes et en dessus de un dans les hémolysats provenant de thalassémies. En revanche, la relation du taux de radio-activité de la leucine-C^{14} était en dessous de un dans tous les échantillons étudiés, même dans ceux provenant de thalassémies. L'interprétation de ces résultats est discutée.

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Thalassaemia in Ceylon

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Thalassaemia has a widespread geographical distribution. It occurs in the Mediterranean populations but has also been found in the Middle East, India and South East Asia and in the people of the Mediterranean stock elsewhere. It represents a genetically inherited disturbance in which haemoglobin formation is inhibited. Haemoglobin has four peptide chains, two pairs being identical. Four different chains have been found in the 3 haemoglobins F, A and A_2 and are referred to as α, β, γ and δ chains. Thus the three normal haemoglobins may be designated as $\alpha_2\beta_2$ (HbA), $\alpha_2\gamma_2$ (HbF) and $\alpha_2\delta_2$ (HbA_2). The basic mechanism of diminished haemoglobin synthesis in thalassaemia is not known. Available evidence is in favour of the hypothesis that thalassaemia is caused by an abnormality in the genetic factors which govern the rate of synthesis of α and β chains of the normal adult haemoglobin. The classical thalassaemia is considered to be caused by diminished production of β chains. Here the synthesis of α, γ and δ chains is not disturbed, hence the relative and absolute amounts of HbF and HbA_2 are increased. On the other hand, if the production of α chain is diminished that of HbF and HbA_2 are also impaired. The excess of β chain tetramerize to form HbH (β_4) an unstable haemoglobin which precipitates. *The variety of thalassaemia accompanied* by HbH or Bart's has been referred to as α-thalassaemia (1).

Clinically the severity of the disorder varies considerably ranging from severe anaemia to that of an asymptomatic trait.

Methods and Materials

The methods used in the haematological investigations have been described in previous communications by Nagaratnam *et al.* (2). Electrophoresis of haemoglobin was carried out using paper at pH 8.6 (veronal) and 8.9 (Tris buffer). Evaluation of HbA_2

fraction was made using control samples of haemoglobin with normal and raised HbA_2. Alkali-denaturation technique of SINGER et al. (3) was used for estimating foetal haemoglobin. Erythrocyte inclusion bodies was demonstrated using brilliant cresyl blue vital staining.

Case Reports

α (Alpha)-Thalassaemia (Thalassaemia-HbH Disease)

Case 1 (Mudiyanse family). A female child (N7) aged 5 months was admitted with history of breathlessness, listlessness and fever. On examination she was febrile, pale and had yellowish discoloration of her skin. The lymph glands were not palpable. The liver was one fingerbreadth below the right costal margin. The spleen was not palpable.

Haemoglobin 2.2 g PVC 9 MCV 75.0 μm^3 MCHC 25.3 WBC 47,000 per mm inclusive of nucleated red cells. DC N 70% L 25 E 4 %, M 1 %. Blood film showed anisopoikilocytosis, hypochromia, nucleated red cells and target cells (Fig. 1). Inclusion bodies were seen in the erythrocytes with brilliant cresyl blue vital staining (Fig. 2). Tests for sickling negative. Serum bilirubin 1.8 mg %. Urine: bile nil, urobilin nil. X-ray of hands tubular bones and skull showed no abnormality. Haemoglobin electrophoresis showed fast moving fraction besides HbA (Fig. 3). Since the blood showed inclusion bodies in the erythrocytes this was probably HbH.

α-Thalassaemia (thalassaemia-HbH disease) was diagnosed. The child was transfused, but was removed from hospital by the parents to be admitted few days later in critical condition and died the following day. No further studies were possible.

Family study (parents and siblings). Clinical examination of the family revealed no abnormality. Alkali resistant Hb ranged from 2.3—4.4 %, highest being in the youngest aged 1 ½ years of the surviving children. No abnormal haemoglobins were detected.

β (Beta)-Thalassaemia

Case 2 (Martin Singho family). A male child (M3) aged 8 months was admitted with history of swelling of his abdomen, pallor and failure to thrive all of one month's duration. On examination, the child was thin and emaciated, was febrile and extremely pale. Examination of the heart revealed systolic murmur haemic in type. The abdomen was distended, the liver and spleen were palpable about 3 fingerbreadths below their costal margins.

Haemoglobin 4.2 g PVC 17 MCV 63.4 μm^3 MCHC 24 %. The blood picture showed anisopoikilocytosis, hypochromia, target cells and large number of nucleated red cells Fig. 4. WBC 210,000 mm inclusive of the large number of nucleated red cells. DC P 30% L 44 E 26 Reticulocytes 6 Total proteins 7.04 g%. Alb glob ratio 0.. Serum bilirubin nil, thymol turbidity 8 units, cephalin cholesterol 2 — Tests for sickling were negative. X rays of the skull, hands and tubular bones showed no abnormality. Alkali-resistant haemoglobin was 36.4 and paper electrophoresis of the sample revealed HbA and HbF.

β Thalassaemia (thalassaemia major) was diagnosed. The child was given blood transfusion and since then had received two more.

Family study (parents and 4 siblings). Mother: Clinical examination revealed no obvious abnormality. The blood picture showed hypochromia and paper electrophoresis revealed raised HbA_2 with no abnormal haemoglobins (Fig. 5). Clinical examination of the rest of the family revealed no abnormality. Alkali-resistant haemoglobin was significantly raised. No abnormal haemoglobin was detected on paper electrophoresis.

Case 3 (Punchi Banda family). A male child aged 8 months was admitted with history of abdominal swelling of two months duration. On examination he was pale



Fig 1 Case 1 Peripheral blood picture showing large number of nucleated red cells in varying stages of maturation.

Fig 2 Case 1 Erythrocyte inclusions in propositus N7 Brillian cresyl blue vital staining. The dense dark bodies in some of the cells are reticulum stained.

and breathless. No lymph glands were palpable The liver was palpable 2 fingerbreadths below the right costal margin nd the spleen 4 fingerbreadths below the left costal margin. No other bnormality was detected

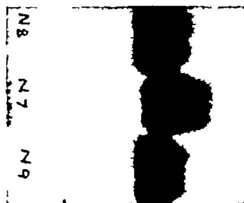
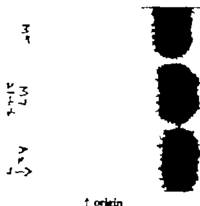


Fig 3. Case 1 Paper electrophoresis showing fast moving fraction of Hb trailing forward in propositus N7



Fig 4. Case 2 Peripheral blood picture showing anisopoikilocytosis, hypochromia, target cells and nucleated red cells.

Haemoglobin 3.3 g PCV 14 MCV 77.7 μm^3 MCHC 23.5 % reticulocytes 6 %, WBC 50,000 mm^3 inclusive of nucleated red cells DC P 34%, L 54 %, E 9%, promyelocytes 3 The blood picture showed anisopoikilocytosis, hypochromia, large number of normoblasts and few target cells. White cells showed shift to the left. Tests for sickling negative Intraerythrocytic inclusions negative. Serum bilirubin 0.2 mg% thymol turbidity 4 units, cephalin cholesterol 1+ zinc sulphate turbidity 16 units. Total proteins 6.0 g% A G ratio 0.62. X ray revealed no abnormality Alkali resistant haemoglobin was found to be 73.5 and electrophoresis revealed single component with the mobility similar to HbF



↑ origin

Fig. 5. Case 2. Paper electrophoresis pH 8.9 (TRIS buffer) showing increased HbA_2 in mother compared with control. The propositus (315) shows HbA + F

β-Thalassaemia (thalassaemia major) was diagnosed. The child was treated with blood transfusions.

Family study: The mother's blood showed normal amount of foetal Hb. (1.27) and on electrophoresis no abnormal haemoglobin was detected. Haemoglobin A_2 was found to be slightly increased.

Thalassaemia with β-Chain Anomalous HbE

The following family was described earlier in detail by one of us (2) and therefore will not be discussed here in detail.

Case 4 (Tennekoon family). A girl aged 13 years was admitted with continued fever, abdominal swelling and dyspepsia. She was pale and apathetic. The liver and spleen were considerably enlarged.

Haemoglobin 2.1 g% PCV 8.5 MCV 150 μm^3 MCHC 25 reticulocytes 17.2%, WBC 7000 mm^3 DC P 68% L 32% Osmotic fragility 0.38% —0.32% The blood picture showed anisopoikilocytosis, polychromasia and basophilic stippling and hypochromia. Serum bilirubin 1.8 mg %. X rays of the skull and tubular bones showed no abnormality; the hands showed rarefaction. No alkali-resistant haemoglobin fraction was detected and paper electrophoresis revealed two components, one with the mobility of HbA and the other of HbE. In spite of failure to detect HbF diagnosis of thalassaemia-HbE disease was made and this had been discussed in a previous communication (2).

Family study. A brother of case 4 aged 10 years was admitted with abdominal swelling of several years duration. The child was pale; the liver and spleen were grossly enlarged. Haemoglobin 6.6 g% PCV 23 MCV 78 μm^3 MCHC 29% reticulocytes 2.8%, WBC 4200 mm^3 DC P 46 L 45 E 9% The blood picture showed anisopoikilocytosis, pessary forms, microcytosis and many target cells and an occasional nucleated red cell. Serum bilirubin 6.8 mg% X rays revealed trabeculations which were marked in the metacarpal bones, phalanges and lower ends of the tubular bones. No changes were seen in the skull. Alkali-resistant Hb was significantly raised (5.0% and paper electrophoresis revealed the presence of HbE and HbF. Thalassaemia-HbE was diagnosed. One other member of the family had thalassaemia HbE disease. The father was regarded as an asymptomatic carrier of HbE trait, and the mother a carrier of thalassaemia trait.

Case 5 (Samel family). A female child aged 6½ years was admitted with swelling of her abdomen, tiredness and loss of appetite all of two months duration. On exam-

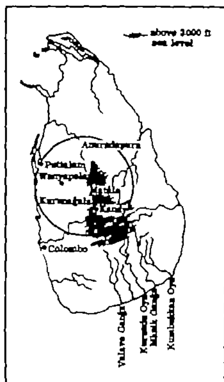


Fig 6 Encircled area indicates regions where the incidence of thalassaemia and HbE is highest.

ination she was dyspnoeic at rest and was extremely pale. She was emaciated and had prominent abdomen. Her liver was palpable 4 fingerbreadths below the right costal margin and her spleen 3 fingerbreadths below the level of her umbilicus.

Haemoglobin 2.1 g% PCV 7% MCV 77 7 μm^3 MCHC 30% reticulocyte 0.5 WBC 8000 mm^3 (inclusive of nucleated red cells) DC P 36%, L 63%, E 1%. The blood picture showed marked anisopoikilocytosis, polychromasia, hypochromia, nucleated red cells and a few target cells. Tests for sickling were negative. Intraerythrocytic inclusions negative. Osmotic fragility 0.38%—0.28%. Liver function tests, serum bilirubin 0.7 mg% thymol turbidity 6, cephalin cholesterol 4+ thymol flocculation 4+ (24 h) zinc sulphate turbidity 15 units. Alkaline phosphate 11 5 KA units. Total proteins 8.0 g% A/G ratio 0.48. X rays of the skull were normal, that of the tubular bones and metacarpals showed thinning of the cortex, decreased density and prominent trabeculations. Haemoglobin electrophoresis showed a major fraction with mobility similar to HbE and the other similar to HbF. Alkali-resistant Hb was 3.6%.

HbE-thalassaemia was diagnosed. The child was treated with blood transfusions.

Family study. Haemoglobin analyses in the case of the father and two other siblings did not show anything abnormal. Alkali-resistant Hb was within normal limits with the absence of HbE in other members of the family including the father. The mother being dead the possibility of the deceased mother being a carrier cannot be ruled out. Haemogram of the father Hb 14.8 g PCV 45 MCHC 33.1 reticulocytes less than 1 no inclusion bodies. The blood picture was normal. Osmotic fragility 0.42%—0.32%

Discussion

In Ceylon there is a mixed population — the Veddahs the Sinhalese the Tamils, the Moors and the Burghers who are of European descent. The Veddahs, the original inhabitants of Ceylon (came from India 6000—3000 B.C.) now occupied the North Central, Uva and the Eastern Provinces. The Sinhalese people came from India about 500 years before the beginning of the Christian era and first settled along the river valleys of the Walave Ganga, Kiruna Oya, Manik Ganga and the Kumbukkan Oya (4) The Tamils came to Ceylon from the earliest times, about the time of the arrival of the Dravidians in South India (3rd and 4th centuries B C.) and settled in the parts now called the Northern and Eastern Provinces. Arab shipping and merchants were established at the port of Colombo as early as 949 (5)

The haemoglobinopathies (thalassaemia and HbE) have so far been described only among the Veddahs and the Sinhalese. GRAFF *et al.* (6) first described the occurrence of HbE in the Veddahs and later LEHMANN (7) found an overall incidence of approximately 10%. HbE has also been described among the Sinhalese (82)

It is not possible to trace the racial origin of HbE in South East Asia. It may have resulted as a mutation. As the Veddahs and the Sinhalese are partly of Bengali extraction HbE in Ceylon probably originated from Bengal where an incidence of 3 9 has been recorded, an incidence similar to heterozygous thalassaemia (9) The majority of the cases are from areas inhabited by Sinhalese of Kandyian origin, and according to SELIGMANN (4) they have a considerable amount of Veddah blood. The encircled area in Fig 6 indicates areas where the incidence of thalassaemia and HbE is highest. The uppermost part of the encircled area includes a part of the dry zone area (Kurunegala and North and Eastern Divisions of the Matale District) which were endemic for malaria for more than 700 years. It is noteworthy that the incidence of HbE seems to be largely confined to areas which were hyperendemic to malaria before 1949 (8) It has been postulated that the Cooley gene probably *originated in Central Asia With race migration they divided* into two lots one moving towards Southern Europe to countries like Greece and Italy and the other moving towards Persia and India. CHERNOFF 10) put forward an attractive hypothesis relative to the distribution of thalassaemia to mass migrations and commerce

which served to carry the genetic defect to China from a single focus in the Northern Mediterranean basin. He also quotes Brumpt to say that this gene arose from India China and moved westwards.

a Thalassaemia (thalassaemia HbH disease) is the rare type of thalassaemia and is due to the inhibition of the α-chain. Thalassaemia HbE disease is the counterpart of this kind of thalassaemia. The abnormal HbH is found only in association with thalassaemia gene and manifests itself as thalassaemia HbH disease. The homozygous HbH nor the carrier state is found Another distinguishing feature of this condition is the finding of intra-erythrocytic inclusions. The mode of inheritance of HbH is still not well understood. The occurrence of HbH was first described by RIGAS *et al* (11) in a Chinese family and is known to occur in the Thais, Filipinos and has been described in two Greek families (12) and in an English family (13)

HbH has not so far been described in Ceylon Though the two distinguishing characteristics, the presence of a fast moving fraction (probably HbH) and intra-erythrocytic inclusions, were seen in case 1 unfortunately further crucial tests could not be carried out. Some members of the family had an increase in the alkali resistant Hb but no abnormal Hb was detected Inclusion bodies without HbH have been reported in Thailand by MINNICH *et al* (14) in three cases presenting as thalassaemia HbH disease Thalassaemia HbH disease varies in severity though it is less severe than thalassaemia major Thalassaemia HbH patients are prone to haemolytic crisis and this is probably what happened to case 1 ending fatally

In *β-thalassaemia* there is besides the increase in the alkali-resistant Hb an increase in the A_2 fraction unlike in α-thalassaemia. The clinical picture of the homozygous form described here differs in no way from that described by DE SILVA *et al.* (8) and others. Symptoms appear within the first year of life, are severe with gross enlargement of the liver and spleen. Majority of them died before the age of two years. The red cells showed increased resistance The blood picture reveals a large number of nucleated red cells in various stages of maturation and a varying number of target cells Radiological changes may be minimal and if found usually occur in the long bones and in the skull. Case 2 showed an increase in HbF Increase in HbF is a well established abnormality in thalassaemia and is regularly found in homozygous forms.

Values are usually high (approximately between 90 —20) Cases 2 and 3 are obviously homozygous forms. The mother of case 3 had an increase in A_2 while the father showed no abnormality

The heterozygotes show considerable individual variation in the expression of the trait. The majority of the cases show little besides mild anaemia with hypochromia and a few or no target cells. In the heterozygous state however HbF may not be significantly raised. Following the identification of A_2 by KUNKEL and WALLENIUS (15) this fraction has become important in the diagnosis of thalassaemia, more so in the heterozygous states (thalassaemia minor and carrier states) where it constantly raised (*vide* family of case 2)

Thalassaemia with β-chain anomolous HbE results from interaction of the thalassaemia gene with the gene for HbE. Like thalassaemia HbH disease the condition presents a wide range of severity as illustrated by cases 4 and 5 Haematological and radiological changes may be indistinguishable from that of thalassaemia major

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Summary

A case of thalassaemia-HbH disease is described for the first time from Ceylon The thalassaemia syndromes in Ceylon now include thalassaemia (major minor and carrier state) thalassaemia-HbH disease and thalassaemia-HbE disease which are discussed

Zusammenfassung

Es wird erstmals ber einen Fall on Thalassämie-Hb H-Krankheit aus Ceylon berichtet. Die Thalassämie Syndrome in Ceylon umfassen nun Thalassämie major minor und Merkmalsträger Thalassämie-Hb H Krankheit und Thalassämie Hb E Krankheit, die diskutiert werden

Résumé

U cas de thalassémie HbH est décrit pour la première fois à Ceylan Les syndromes thalassémiques comprennent actuellement à Ceylan la thalassémie majeure mineure et les états de porteurs de gènes ainsi que les maladies thalassémiques vec HbH et HbE qui sont discutées ici.

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Essentielle Hyperlipämie und Blutgerinnungsfaktoren

F. HOLZKNECHT und H. BRAUNSTEINER

Bei der Prüfung von Zusammenhängen zwischen Störungen des Fettstoffwechsels und ihrem Einfluß auf die Blutgerinnung, bei des im Hinblick auf koronarsklerotische Erkrankungen, war einerseits eine vermehrte Aggregationsneigung der Blutplättchen (26), andererseits eine Erhöhung des Fibrinogenspiegels mit einer Verminderung der Fibrinolyseaktivität essentieller Hyperlipämiker (32) gegenüber Normalpersonen an unserer Klinik gefunden worden. MCDONALD *et al.* (20) sowie JAMES *et al.* (15) fanden bei Patienten mit koronarsklerotischen Erkrankungen eine vermehrte Gerinnungsfähigkeit des Blutes im Thrombokinasebildungstest (TGT) sowie bei der Bestimmung der Prothrombin- bzw. der Stypvenzeit. EGEBERG (7) berichtete nur über eine signifikante Vermehrung der Faktor VIII-Aktivität. Die Verabreichung einer atherogenen Diät an Ratten führte nach MERSKEY *et al.* (22) zu einer Erhöhung der Faktoren I, II, V, VII/X, VIII, IX und X. SCOTT (31) hingegen stellte dabei eine Hypokoagulabilität des Blutes *in vivo* fest.

Bei essentieller Hyperlipämikern hatten NITZBERG *et al.* (23), PEYMAN *et al.* (24) ebensowenig wie KOMMERELL *et al.* (17), der die Rekalzifizierungszeit, die Thromboplastinzeit und die Faktoren V und VII prüfte, eine Vermehrung der Gerinnungsaktivität gefunden.

Die unterschiedlichen Angaben über das Verhalten der Blutgerinnungsfaktoren bei gestörtem Fettstoffwechsel, auch experimenteller Art, waren der Anlaß, bei unserem Krankengut von essentiellen Hyperlipämikern auch die plasmatischen Faktoren bzw. Inhibitoren der Gerinnung sowohl in Gruppen als auch, soweit methodisch möglich, in Einzeltests zu prüfen und mit Normalwerten zu vergleichen.

Methodik

Bei 11 Patienten im Alter zwischen 35 und 64 Jahren mit einer essentiellen Hyperlipämie (Triglyzeride im Serum zwischen 410 und 6425 mg%) und 10 Normalpersonen mit normalen Triglyzeridwerten (4) wurden morgens etwa 16 Stunden nach der letzten Nahrungsaufnahme 18,0 ml Blut mit innenpolierter Flügelnadel aus der Armvene in einem Plastikröhrchen, in dem 2,0 ml Natrium citricum tribasicum 3,64% vorgelegt waren, aufgefangen, daraus die übliche Thrombelastographie angesetzt und das Plasma nach Zentrifugieren mit 3000 rpm durch 10 min zur Bestimmung der Gerinnungsfaktoren abpipettiert. Die nächsten 8 Tropfen Blut kamen direkt aus der Flügelnadel in eine Plastikküvette zum Sofortansatz der „Langzeit-Thrombelastographie". Außerdem wurden ca. 10,0 ml Blut zur Gewinnung von Serum in einem nicht silikonisierten Röhrchen mit 5 Glasperlen gesammelt. Aus dieser Flügelnadel wurde weiter Blut zur Bestimmung der Fettwerte (28) in einem heparinisierten Plastikröhrchen entnommen und sofort nachher bei 3000 rpm und 4 °C 10 min lang zentrifugiert. Das Plasma wurde abgehebert und unmittelbar darauf nach der Methode von FOLCH *et al.* (11) in der Modifikation von CARLSON (5) extrahiert. Die Bestimmung der Triglyzeride erfolgte aus dem Extrakt nach CARLSON (5).

Es wurden folgende Gerinnungsbestimmungen durchgeführt:

A. Gesamt- (Global-) bzw. Gruppentests

1. Die *Thrombelastographie* nach HARTERT (12) wurde in zweifacher Weise angewendet. Einmal als Nativblut-Thrombelastographie mit einer Laufgeschwindigkeit der Filmrolle von 2 mm/25 mm (= 1:25 gegenüber der üblichen Laufgeschwindigkeit) als „Langzeit-Thrombelastographie". Es wurde die Kurvenamplitude in mm nach 1 h (= 4,8 mm Filmband) und nach 24 h (= 115,2 mm Filmband) bestimmt. Die Differenz beider Amplitudenwerte in mm erscheint als ein Maß für die in 23 h stattgehabte Fibrinolyse. Zum Zweiten erfolgte die konventionelle Thrombelastographie mit Zitrat-Vollblut in Plastikküvetten in üblicher Weise (Laufgeschwindigkeit des Filmbandes 2 mm/min). Die Auswertung erfolgte nach den bekannten Parametern r = Reaktionszeit in Minuten, k = Gerinnselbildungszeit in Minuten und Ma = maximale Amplitude in mm.

2. *Partial Thromboplastin Time Test* (PTT nach RODMAN *et al.*, 27). An Stelle einer Kephalinsuspension wurde zur Prüfung des endogenen Gesamt-Gerinnungsvorganges nach den Angaben von DE NICOLA *et al.* (6) sowie FISCHER (10) Tachostyptan (Hormonchemie, München) verwendet. Aus je 0,1 ml Zitratplasma, 0,1 ml Tachostyptanlösung 1:50 in Veronalpuffer (Michaelis) pH 7,4 und 0,1 ml m/40 $CaCl_2$-Lösung wurde die Gerinnungszeit bestimmt (Kippmethode im Röhrchen, Wasserbad bei 37 °C). Dieser Test wurde sowohl in nicht-silikonisierten als auch in silikonisierten Glasröhrchen durchgeführt. Die Differenz beider Gerinnungswerte in Sekunden wurde als Maß für die Aktivität der Kontaktfaktoren angesehen, so daß eine Verlängerung der Gerinnungszeit nur im nichtsilikonisierten Röhrchen – mithin eine Verkleinerung der Differenz zwischen den Werten in den beiden Röhrchen – als Aktivitätsverminderung der Kontaktfaktoren angesehen wurde.

3. Der *Quick-Test* (25) dient der Bestimmung der Aktivitäten der Faktoren I (Fibrinogen), V (Proaccelerin), VII (Proconvertin) und X (Stuart-Prower-Factor). Der Test wurde in üblicher Weise mit Zitratplasma im Wasserbad bei 37 °C durchgeführt und dabei Thrombokinase-Calcium-Tabletten (Geigy, Basel) verwendet.

4. Mit dem *Stypven-Lecithintest* (21) wird hauptsächlich die Aktivität des Prothrombins und Faktors X (Stuart-Prower-Factor) erfaßt. Zuerst wird ein Gebrauchsreagens zubereitet. Es enthält 0,2 ml Stypvenlösung (0,5 mg Russell Viper Venom der Firma Burroughs Wellcome & Co., London, werden mit den 5,0 ml des beigegebenen Lösungsmittels aufgelöst), 1,8 ml Aq. dest., 2,0 ml Lecithin-Suspension (15,0 mg Leci-

thin et ace Merck werden in 100,0 ml warmem Aq. dest. suspendiert) und 4,0 ml m/40 $CaCl_2$-Lösung. Zu 0,1 ml Zitratplasma kommen 0,2 ml Gebrauchsreagens, beide etwa bei 37 °C vorgewärmt. Die Gerinnungszeit wird im Röhrchen durch Kippen bestimmt. Die Aktivität des Prothrombins und des Faktors X wurde in Prozenten von einer Eichkurve abgelesen, die aus 100%- sowie 50%-, 25- und 12,5%-Verdünnungen von Normalplasma mit Diäthyl-Barbiturat-Acetat-Puffer (Behringwerke) pH 7,6 erstellt wurde.

B. «Einzel-Faktoren-Tests»

1. Die *Faktoren V (Proaccelerin) und VII (Proconvertin)* wurden in einem Einphasentest mit den Faktor V- und Faktor VII-Reagens Behringwerke durchgeführt (29, 30).

2. *Faktor II (Prothrombin).* Die Bestimmung des Prothrombins (Faktor II) erfolgte nach der Zweiphasen-Methode ohne Zusatz von Acceleratoren mit Affenhirnthrombokinase. Das Zitratplasma wurde 1:30 mit physiologischer NaCl-Lösung verdünnt. Die kürzeste Gerinnungszeit wurde als Maß für die Prothrombinaktivität aufgefaßt (29, 30).

3. *Faktor VIII (antihämophiles Globulin).* Die Bestimmung erfolgte in einem Zweiphasentest nach Biggs et al. (2), modifiziert nach Larrieu (19). Für das Ansatzgemisch wurden zu 0,1 ml Normalserum, 1:10 mit Veronalpuffer (Michaelis) pH 7,4 verdünnt, und an Stelle von Kephalin Tachostyptan (Hormonchemie München) 0,1 ml 1:50 ebenso verdünnt dazugegeben, 60 min bei 37 °C im Wasserbad inkubiert und dann in Eiswasser gestellt. *In der ersten Phase wurden zu jeweils 0,2 ml dieses Ansatzgemisches* je 0,1 ml 1:20 und 1:40 mit Veronalpuffer (pH 7,4) verdünntes $Al(OH)_3$-adsorbiertes Zitratplasma (Testplasma), ferner 0,1 ml an $Al(OH)_3$ adsorbiertes Faktor VIII-Mangelplasma mit einem Titer unter 1% als Faktor V-Spender 1:10 verdünnt mit Veronalpuffer (pH 7,4) sowie 0,1 ml $CaCl_2$ m/40 hinzugegeben (Inkubationsgemisch) und die Stoppuhr in Gang gesetzt. Für die zweite Phase wurden in mehr als 5 Röhrchen 0,1 ml $CaCl_2$ m/40 vorgelegt. Alle 2 min wurden aus dem Inkubationsgemisch der ersten Phase 0,1 ml sowie 0,1 ml Zitrat-Normalplasma (nicht adsorbiert) zum vorgelegten $CaCl_2$ hinzugegeben und die Gerinnungszeit bestimmt. Die kürzeste Gerinnungszeit wird zur Berechnung der Faktor VIII-Aktivität herangezogen. Die Eichkurve zur Ablesung der Faktor VIII-Aktivität in Prozenten wird aus 1:20 (100%), 1:40 (50%), 1:80 (25%) und 1:160 (12,5%) Verdünnungen von $Al(OH)_3$-adsorbiertem Normalzitratplasma nach derselben Methode erstellt und auf doppelt logarithmischem Papier aufgetragen. Die Prozentwerte werden auf der Abszisse, die Gerinnungszeiten in Sekunden auf der Ordinate eingetragen. Bei der Ermittlung der Faktor VIII-Aktivität wird zuerst der Prozentsatz aus der Plasmaverdünnung 1:20 ermittelt. Dann erfolgt derselbe Vorgang für die Plasmaverdünnung 1:40. Dieser Prozentwert wird verdoppelt, dem Prozentwert für die 1:20 Verdünnung hinzugerechnet und das Mittel gebildet, das der endgültigen Faktor VIII-Aktivität entspricht.

4. *Faktor IX (Christmas-Factor).* Die Bestimmung erfolgte nach dem Einphasentest von Biggs et al. (3), modifiziert nach Larrieu (19). Zu 0,1 ml Test-Zitratplasma-Verdünnung 1:40 und 1:80 mit Veronalpuffer (pH 7,4) wurden 0,1 ml Faktor IX-Mangelplasma (Titer unter 1%) sowie 0,1 ml Kaolinsuspension (20 mg/ml Veronalpuffer pH 7,4) hinzugegeben und das Gemisch bei 37 °C im Wasserbad durch 2 min inkubiert. Dann erfolgte die Zugabe von 0,1 ml Tachostyptanlösung 1:50 in Veronalpuffer pH 7,4, *an Stelle einer Kephalinsuspension,* sowie 0,1 ml m/40 $CaCl_2$-Lösung. Die Gerinnungszeit wurde gestoppt. Die Aktivität an Faktor IX wurde von einer Eichkurve abgelesen, die aus Ansätzen von 1:40 (100%), 1:80 (50%), 1:160 (25%) und 1:320 (12,5%) Verdünnung Normalplasma erstellt und auf doppelt logarithmischem Papier wie oben aufgetragen worden war. Aus dem Prozentwert für die Verdünnung

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1:40 und dem doppelten Wert für die Verdünnung 1:80 wird die Summe gebildet und durch 2 geteilt. Dieser Wert entspricht der Faktor IX-Aktivität des geprüften Zitratplasmas.

C. Inhibitoren der Blutgerinnung

1. *Blutantithrombokinase.* Die Bestimmung wurde nach der Methode von EGLI *et al.* (9) in modifizierter Weise durchgeführt. Zuerst erfolgte die Herstellung der Blutthrombokinase nach dem Thrombokinasebildungstest (2), wobei wir jedoch an Stelle der Thrombozytensuspension eine Tachostyptanlösung (Tachostyptan, Hormonchemie, München) 1:50 mit Veronalpuffer pH 7,4 verdünnt verwendeten. Sobald die Blutthrombokinase (nach 2 min Inkubation) ihre maximale Aktivität erlangt hatte, wurde sie in Mengen von 1,5 ml in silikonisierten Röhrchen bei −20 °C eingefroren.

Zur Bestimmung der Blutthrombokinase-Inaktivierung (als Ausdruck der Antithrombokinase-Aktivität) wurde die zu 1,5 ml eingefrorene Blutthrombokinase aufgetaut und 1,5 ml Testserum der Normalperson bzw. des Patienten hinzugegeben und die Gerinnungszeit bestimmt. Die Inkubationszeit wurde auf der Abszisse, die Gerinnungszeit auf der Ordinate eingetragen. Eine verminderte Aktivität der Blutantithrombokinase wäre an einer zunehmend kürzeren Gerinnungszeit gegenüber Normalplasma zu ersehen. Für unsere Untersuchungen verglichen wir die Differenzen in Sekunden zwischen den 1., 5., 9. und 12. min Inkubation der Normalpersonen und Hyperlipämiker.

2. *Antithrombin II.* Die Bestimmung erfolgte nach JÜRGENS *et al.* (16). Zu 0,1 ml Zitratplasma werden 0,1 ml Aq. dest. und 0,1 ml einer Thrombinlösung (6 Einheiten Thrombin/ml von Testthrombin Behringwerke, gelöst in Aq. dest.) hinzugegeben und die Gerinnungszeit im Wasserbad bei 37 °C bestimmt.

3. *Antithrombin III.* Die Bestimmung wurde nach der Methode von HENSEN *et al.* (13) vorgenommen. Eine Thrombinlösung mit 50 Einheiten/ml, gelagert bei −25 °C, wurde aufgetaut und in schmelzendes Eis gestellt. Zu 0,2 ml dieser Thrombinlösung in einem silikonisierten Röhrchen wurden 0,6 Veronalpuffer pH 7,4 hinzugegeben und das Röhrchen ins Wasserbad bei 37 °C gestellt. Nach Vorwärmen durch 2 min wurden 0,2 ml hitzedefibriniertes Zitrattestplasma hinzugegeben und die Stoppuhr gestartet. Genau nach 1, 3 und 5 min wurden aus diesem Inkubationsgemisch 0,2 ml zu 0,3 ml $BaSO_4$-adsorbiertem Rinderzitratplasma, das in nicht-silikonisierten Röhrchen durch 2 bis 20 min vorgewärmt war, hinzupipettiert und jeweils die Gerinnungszeit bestimmt. Dabei wurden also 3 Gerinnungszeiten ermittelt. Entsprechend einer Standardkurve (Gerinnungszeiten von Normalplasmen nach derselben Methode gegenüber einer Thrombinverdünnungsreihe von 50 Einheiten Thrombin bis 3 Einheiten/ml) wurde für jede ermittelte Gerinnungszeit das Äquivalent Thrombineinheiten ermittelt. Ausgehend von den 3 Werten in Thrombineinheiten wurde der jeweils verbleibende Thrombinrest nach 3 und 5 min in Prozenten des Ausgangswertes errechnet. An Hand einer Standard-Bezugskurve, die aus 100 bis 12,5% Antithrombin-III-Aktivitäten (Verdünnungsreihe von Normalplasmen nach derselben Technik) und den Prozentsätzen Rest-Thrombin nach den 2 min Inkubation (letztere Werte auf logarithmischer Achse) erstellt wurde, liest man nun für die beiden Werte Rest-Thrombin den äquivalenten Prozentsatz Antithrombin-III-Aktivität ab und bildet das Mittel.

Die Ergebnisse wurden statistisch ausgewertet. Falls eine Streuungshomogenität gesichert war, wurde der t-Test nach STUDENT, im anderen Falle der WILCOXON-Test (W) angewendet. Die Aufschlüsselung der Ergebnisse ist aus den Tabellen I bis III ersichtlich.

Ergebnisse

A. Gesamt- (Global-) bzw. Gruppentests (Tab. I). Die übliche Thrombelastographie für Zitratvollblut nach HARTERT (12) ergab

Tabelle 1

Gesamt-(Global-) bzw. Gruppentests der Blutgerinnungsfaktoren bei Normalpersonen und essentiellen Hyperlipämikern.

Methode	n	$\bar{x}$		Signifikanz
TEG 2 mm/1 mm	10 (9)			
	10 (9)	9,9 (13,5)	± 4,2 (3,3)	FG = 17 t = 2,08 p < 0,05
k	10 (9)	5,1 (7,5)	± 2,1 (1 7)	FG = 17 t = 2,76 p < 0,01
Ma	10 (9)	52,9 (46,5)	± 5,2 (4 1)	FG = 17 t = 3,1 p < 0,005
TEG 2 mm/25 mm	7 (9)			
D 1/24 h		17 7 (22,0)	± 4,5 (5,7)	FG = 14 t = 1 73 p < 0,1
PTT Sekunden				
nicht silikonisiert	11 (9)	98,2 (78,5)	±22,6 (20,6)	FG = 18 t = 2,14 p < 0 025
silikonisiert	10 (8)	137,5 (119 1)	±25,8 (28,4)	FG = 16 = 1,51 p < 0,1
D nicht silik. — silik.	10 (8)	39,8 (44 4)	±12,8 (26,0)	nicht-sgf. (W)
Quick-Test, Sekunden	10 (10)	18,7 (19,4)	± 1,3 (2,0)	FG = 18 t = 0,89 p < 0,2
Styptven-Lecithintest, Sekunden	11 (10)	22,2 (20,1)	± 3,1 (3,4)	FG = 19 = 1,52 p < 0,1

Zeichenerklärung: D = Differenz, FG = Freiheitsgrade, = Anzahl der Fälle, p = Signifikanzniveau, PTT = Partial Thromboplastin Time, = Streuung, = t-Test, TEG = Thrombelastogramm mit den Parametern = Reaktionszeit in Minuten, k = Gerinnselbildungszeit in Minuten, Ma = Maximal-Amplitude in mm, $\bar{x}$ = Mittelwert, W = Wilcoxon Test (sgf. = signifikant, nicht-sgf. = nicht-signifikant)

Unterstrichene Werte sind signifikant, Werte außerhalb der Klammern entsprechen den essentiellen Hyperlipämikern, in den Klammern den Normalpersonen.

für die Parameter r (Reaktionszeit) und k (Gerinnselbildungszeit) in Minuten eine Beschleunigung der Gerinnungsfähigkeit des Blutes der Hyperlipämiker für die Ma (Maximale Amplitude) in mm einen erhöhten Wert gegenüber dem Blut von Normalper

Tabelle II
«Einzel-Faktoren Tests» der Blutgerinnung bei Normalpersonen und essentiellen Hyperlipämikern.

Methode	n	$\bar{x}$	s	Signifikanz
Faktor V (Proaccelerin) Sekunden	11 (10)	35,2 (33,9)	±1,8 (1,6)	FG = 19 t = 1,8 p < 0,05
Faktor VII (Proconvertin) Sekunden	10 (10)	29,4 (31,3)	±1,2 (1,6)	FG = 18 t = 2,8 p = 0,01
Faktor II (Prothrombin) Sekunden	8 (9)	16,3 (15,2)	±2,1 (1,1)	FG = 15 t = 1,4 p < 0,1
Faktor VIII (antihämophiles Globulin) Sekunden	8 (9)	13,5 (12,5)	±0,5 (1,1)	sgf. (W)
Faktor IX (Christmas-Factor) Sekunden	7 (9)	95,4 (95,5)	±7,9 (8,5)	nicht sgf. (W)

Zeichenerklärung FG = Freiheitsgrade, = Anzahl der Fälle p = Signifikanzniveau, = Streuung = t Test, $\bar{x}$ = Mittelwert, W = Wilcoxon Test (sgf. = signifikant)

Unterstrichene Wert sind signifikant, Werte außerhalb der Klammern entsprechen den essentiellen Hyperlipämikern, in den Klammern den Normalpersonen.

sonen. Das Ergebnis ist statistisch deutlich gesichert. Bei der Thrombelastographie mit langsamer Übersetzung (1 25) ist der Unterschied in den Differenzwerten der Maximalamplitude in mm gemessen nach 1 und 24 h, nicht von der erforderlichen Signifikanz

Demgegenuber zeigt die Partialthromboplastinzeit in Sekunden in nicht-silikonisierten Rohrchen bei den essentiellen Hyperlipämikern einen gesichert verlängerten Wert gegenüber Normalpersonen In den silikonisierten Rohrchen und in der Höhe der Differenz der Gerinnungszeiten zwischen nicht silikonisiertem und silikonisiertem Ansatz konnte ein sicherer Unterschied in der Gerinnungszeit nicht verifiziert werden

Im Quickwert ist ein Unterschied zwischen beiden Kollektiven nicht zu sichern ebensowenig im Stypven Lezithin Test.

B «Einzel Faktoren Tests» (Tabelle. II) Für die Absolutwerte in Sekunden zeigt der Faktor VII (Proconvertin) eine gesichert vermehrte Aktivität bei den essentiellen Hyperlipämikern gegen-

Tabelle III

Inhibitoren der Blutgerinnung bei Normalpersonen und essentiellen Hyperlipämikern.

Methode	n	$\bar{x}$		Signifikanz
Blutantithrombokinase, Sekunden				
D zw 1 und 5 min	10 (9)	9,2 (9,9)	±3,7 (4,5)	nicht sgf. (W)
D zw 5 und 9 min	10 (9)	13,7 (10,7)	±4,4 (9,0)	nicht sgf. (W)
D zw 9 und 12 min	10 (8)	8,1 (2,4)	±7,0 (7,5)	nicht sgf. (W)
Antithrombin II, Sekunden	11 (10)	19,1 (18,1)	±2,7 (2,4)	FG = 19 t = 0,93 p < 0,2
Antithrombin III, Sekunden				
D zw 1 und 3 min	11 (9)	5,2 (3,7)	±1,1 (1,8)	sgf. (W)
D zw 3 und 5 min	11 (9)	6,6 (8,2)	±1,8 (3,5)	nicht sgf. (W)

Zeichenerklärung: D = Differenz, FG = Freiheitsgrade, n = Zahl der Fälle, p = Signifikanzniveau, = Streuung, $\bar{x}$ = Mittelwert, W = Wilcoxon Test (sgf. = signifikant)

Unterstrichene Werte sind signifikant, Werte außerhalb der Klammern entsprechen den essentiellen Hyperlipämikern, in den Klammern den Normalpersonen.

über den Normalpersonen, die Aktivität der Faktoren VIII (antihämophiles Globulin) und Faktor V (Proaccelerin) hingegen ist sicher vermindert. Die Aktivitäten in Prozentwerten gegenüber den Normalwerten von jeweils 100% betrugen für Faktor VII im Mittel 130% für die Faktoren VIII 45,5% und V 85%. Beim Faktor IX (Christmas Factor) und Faktor II (Prothrombin) konnte kein signifikanter Unterschied ermittelt werden.

C. Inhibitoren der Blutgerinnung (Tabelle III). In der Entwicklung der Blutantithrombokinase sowie beim Antithrombin III war kein Unterschied zwischen den essentiellen Hyperlipämikern und den Normalpersonen zu sichern. Beim Antithrombin III konnte am Beginn der Antithrombinaktivierung (zwischen der 1 und 3 min) bei den Hyperlipämikern eine Verzögerung des Aktivierungsvorganges statistisch gesichert werden, nicht aber zwischen der 3 und 5. min der Inkubation. Bei Umrechnung der Absolutwerte des Antithrombin III entsprechend der Eichkurve ergibt sich gegenüber dem Normalkollektiv mit einer Antithrombinaktivität von 100 / bei den Hyperlipämikern ein Wert von 83,5 %

Diskussion

Die Prüfung der Blutgerinnungsfaktoren in Gesamt (Global-) Gruppen und Einzeltests sowie von Inhibitoren der Blutgerinnung bei essentiellen Hyperlipämikern (Triglyzeridwerte zwischen 410 und 6425 mg%) im Vergleich zu Normalpersonen zeigte bei statistischer Auswertung keinen einheitlichen Trend zu einer Hyperkoagulämie bei der ersten Gruppe. Dies deckt sich mit den Angaben von KOMMERELL *et al.* (17)

Die Prüfung der Gesamtgerinnung nach der Thrombelastographie HARTERT (12) ergibt eine signifikante Verkürzung bei den Hyperlipämikern. Sie kann im gegebenen Ausmaß wohl kaum der bei dieser Untersuchung gefundenen Aktivitätssteigerung des Faktors VII und der Verminderung des Antithrombins III zugeschrieben werden, da bei beiden die Veränderung zwar signifikant, jedoch *für eine Gerinnungswirkung zu geringfügig sein dürfte. Vielmehr* muß daran gedacht werden, daß die in zwei vorangegangenen Studien (26, 32) gefundene verminderte Fibrinolyse und gesteigerte Aggregationsneigung der Thrombozyten dafür verantwortlich zu machen ist.

Im Gegensatz dazu stehen die verlängerte Partial Thromboplastin Zeit sowie die verminderte Aktivität von Faktor V und Faktor VIII SCOTT (31) fand bei seinen Versuchen an Ratten durch atherogene Diät zwar die Entwicklung einer Hyperlipämie und Atherosklerose, jedoch *in vivo* eine Hypokoagulabilität. KOMMERELL *et al* (17) sahen bei Lipofundingaben bei Kaninchen Gerinnungsbefunde, die sie vor allem nach Zugabe von Tusche zur Blockierung des RES als Folge einer «Verbrauchskoagulopathie» auslegten. In diesem Sinne könnten auch unsere Befunde gewertet werden, da ja beim Gerinnungsvorgang gerade die hier vermindert gefundenen Faktoren V und VIII verbraucht werden.

Bei den Versuchen von KOMMERELL *et. al.* (17) würde allerdings der angegebene Aktivitätsverlust von Faktor VII nicht ganz zu dieser Annahme passen er wird ja beim Gerinnungsvorgang nicht verbraucht, er ist bei unseren Ergebnissen auch nicht vermindert, sondern vermehrt.

Die verlängerte Partial-Thromboplastin-Zeit, die im Widerspruch zu den Ergebnissen der Thrombelastographie zu stehen scheint, ließe sich mit Vorsicht so deuten, daß sie im Gegensatz zur Thrombelastographie mit thrombozytenarmem Plasma durch-

geführt wird und auch eine verminderte Fibrinolyse dabei kaum zum Ausdruck kommen kann. Sie könnte vor allem durch den Mangel an Faktor VIII beeinflußt sein.

Eine pathophysiologische Erklärung für diese hier milde Form einer «Verbrauchkoagulopathie» könnte in Anlehnung an KOMMERELL *et al.* (17) darin liegen, daß in unserem Falle bei den Hyperlipämikern etwa auch ohne Blockierung des RES durch Tuschegaben, die Klärwirkung des RES auf die Gerinnungsfaktoren durch die Hyperlipämie gestört wird (33).

Beim Faktor II (Prothrombin), beim Faktor IX (Christmas-Factor), beim Stypven-Lecithintest (Faktoren II und X) und beim Quicktest konnte keine signifikante Veränderung gefunden werden. Dasselbe gilt für eine Änderung der Kontaktwirkung, die mit dem PTT in silikonisierten Röhrchen und der Differenz aus beiden PTT Ansätzen geprüft wurde.

EGGSTEIN (8) fand beim Vergleich von Normalpersonen mit Patienten, die vermehrte Blutfettwerte aufwiesen, nach Heparingaben eine beschleunigte Rückkehr des Antithrombin II zur Norm bei der Patientengruppe, weswegen bei ihnen auf eine verminderte Heparintoleranz geschlossen wurde. Dies konnten wir in einer früheren Studie bei etwas anders gelagerter Versuchsanordnung bestätigen (14). Bezüglich der Ausgangswerte an Antithrombin II fanden wir damals wie auch EGGSTEIN (8) keinen Unterschied zwischen Normalpersonen und Hyperlipämikern. In Ergänzung zu den damaligen Untersuchungen ergab sich auch diesmal kein signifikanter Unterschied des Antithrombin II bei den Hyperlipämikern gegenüber Normalpersonen.

Wir danken der Techn. Ass. BRIGITTE [illegible] für ihre wertvolle Hilfe bei der Durchführung der Gerinnungsbestimmungen.

Zusammenfassung

Bei 11 essentiellen Hyperlipämikern und 10 Normalpersonen wurden die Gerinnungsfaktoren und Inhibitoren vergleichend geprüft. Während sich bei der üblichen Thrombelastographie eine signifikante Verkürzung der Gerinnung zugunsten der Hyperlipämiker ergibt, zeigen Gruppen- bzw. Einzeltests ein unterschiedliches Verhalten der geprüften Faktoren. Im Quicktest, Stypven-Lecithin Test und bei den Faktoren II und IX, ferner bei der Fibrinantithrombokinase und beim Antithrombin II ergibt sich kein Unterschied. Eine signifikante Verminderung der Faktoren V und VIII wird als mögliche Folge einer leichten Verbrauchskoagulopathie diskutiert, die in der verlängerten Partial Thromboplastin-Zeit ihren Ausdruck findet.

Summary

Blood clotting factors and inhibitors were compared in eleven subjects with essential hyperlipaemia and ten normal persons. Whilst the usual method of thrombelastography showed a significant reduction of clotting time in hyperlipaemic subjects, group and individual tests indicate that the factors tested do not behave uniformly. No difference was seen in the Quick test, Stypven lecithin test and in factors II and IX, or in blood antithrombokinase and antithrombin II. A significant reduction in factors V and VIII is discussed as being possibly the result of a coagulation defect due to attrition and manifested in the prolonged partial thromboplastin time.

Résumé

Les facteurs et les inhibiteurs de la coagulation ont été étudiés comparativement chez 11 personnes atteintes d'hyperlipémie essentielle et chez 10 personnes normales. La thrombélastographie mit en évidence chez les hyperlipémiques un raccourcissement de la coagulation significative; les tests globaux et les tests individuels montrent un comportement variable des facteurs examinés. Le test de Quick, celui de la stypven-lécithine, la détermination des facteurs II et IX, ainsi que celle de l'antithrombokinase sanguine et de la thrombine II ne révélèrent aucune différence. Une diminution significative des facteurs V et VIII est interprétée comme étant la suite possible d'une légère coagulopathie par consomption, coagulopathie qui se manifesterait par un temps partiel de thromboplastine prolongé.

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A Study of Various Antibodies and Genetically Determined Serum Groups among Aborters Producing Anti Tj[a]-Like Hemolysis and Non Aborters in Western Australia*

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Since the discovery of the anti-Tj like hemolysin (7) extensive investigations have been carried out attempting to relate this factor to the obstetric history of the mother the various blood groups of the red cells, and other known types of hemolysins (9 10)

The possibility that the West Australian aborters are immunologic hyper responders, and thus more often form other types of antibodies as well as hemolysins seemed to warrant investigation. In addition, it also seemed of importance at this stage to assess the possible association of various genetically determined serum groups which may be associated with the occurrence of anti-Tj like hemolysins.

The present report is therefore mainly concerned with the study of two parameters measurable in the serum (1) The distribution of antibodies reactive against known red cell antigens, γ-globulin factors (SNagg agglutinators) and C-reactive protein, the latter being included as a possible indicator of diseased conditions, and (2) the comparative evaluation of genetically determined Gm, Inv haptoglobin and transferrin serum groups among aborters and nonaborters A brief summary is also presented in connection with the composition of the various nationalities to establish the extent of racial difference among mothers sampled in this study

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Materials and Methods

Cases for investigation. The sera of the mothers of the abortion and nonabortion (control) groups were in all instances obtained during the antenatal stage of pregnancy. The classification of 'abortion series' was dependent on the clinical observation that the conception threatened to abort, together with the occurrence of anti-Tj^a-like hemolysins, while the 'nonabortion series' included mothers with at least two or more normal pregnancies with no history of threatened abortion. With respect to age distribution, period of sampling of blood and socio-economic background the abortion and nonabortion series of mothers can be considered as comparable.

Red cell antibody investigation. Heat-inactivated serum samples were tested by the indirect antiglobulin, ficin, and saline agglutinating procedures at 22 and 37 °C against a selected series of 6 group 0 red cells known to possess antigens C^W C, D, c, e, Kk, Fy^a Jk^a Jk^b Le^a Le^b MNSs, P and P_k. Sera producing agglutination of one or more of the known red cells were recorded as positive. No distinction was made to identify the specificity of the observed antibody in the event that positive test was recorded.

C-reactive protein test. For the detection of C-reactive protein the tests were performed according to the directions supplied by the manufacturers. The reagents were generously donated by the DIFCO Laboratories, Detroit.

Gm serum groups. The reagents used for the determination of the Gm serum groups were as follows: (1) For Gm( ) agglutinator Higham at dilution of 1 20 and anti-Rh Bunting t a dilution of 1 10 (2) for Gm(b) agglutinator Bomb at dilution of 1:16 or monkey serum immunized for Gm(b) t dilution of 1 10 and anti-Rh Knecbone at dilution of 1 3 (3) for Gm(x) agglutinator Bowers at a dilution of 1 30 and anti-Rh Kemp t dilution of 1 10. The serum samples of the aborters and nonaborters were, for the determination of their Gm types, inactivated at 56 °C for 20 minutes and tested at dilutions of 1 6 and 1 12.

Inv serum groups. Inv (1) typing was performed using agglutinator Herzog at 1·4 and anti-Rh Clark t a dilution of 1:20.

Determination of haptoglobin and transferrin serum groups. Haptoglobin and transferrins were studied according to the vertical starch gel electrophoretic method of Smithies (5). Hemoglobin was added to the sera to saturate the haptoglobins. As usual, the gel was sliced into two parts, one of which was stained with amino black to detect transferrins, and the other with benzidine to visualize the hemoglobin-haptoglobin complex. A number of the samples were restudied for transferrins by the autoradiographic method of GIBLETT *et al.* (2) using iron [59] Deficiency in haptoglobin and ahaptoglobinemia were evaluated from the starch gel, and the examination was repeated three times with concordant results in each serum designated deficient or ahaptoglobinemic.

Anti-Tj^a-like hemolysin test. The technique used for the determination of this hemolysin has been described in previous publications (7 10)

Results

Table I shows a highly significant variation between the frequency of hemolysins (anti Tj^a like and immune anti-A_1) observed among the control and abortion series of mothers examined in this study Since previous reports have not detailed the distribution of the various nationalities, a summary of their composition is included in Table II. It can be seen that at least 79% of the mothers examined were of British nationality and a significantly greater

Table I

The frequency of immune anti-A, anti-B and anti-TJ^a-like hemolysins observed for the control and abortion series of mothers examined during pregnancy

Test Immune hemolysins	Control series No. examined	Control series Present	Abortion series No. examined	Abortion series Present	Chi-square (Yates correction applied)
A_1	204	18	288	86	$\chi^2(1)$ = 30.455 (P = 0.001)
B	204	7	288	15	$\chi^2(1)$ = 0.516 n.s.
TJ^a-like	204	0	288	222	$\chi^2(1)$ = 283.440 (P = 0.001)

Table II

The distribution of various nationalities observed among the control and abortion series of mothers

Nationality (by birth)	Control series No. examined	Control series Observed		Abortion series No. examined	Abortion series Observed		Chi-square (Yates correction applied)
British	204	147		288	239		$\chi^2(1)$ 7.802 (P = 0.01)
Others*		57			49		
Includes							
Italian		34 = 59%			28 = 57%		
Dutch		9			9		
Spanish		3			4		
Greek		4			4		
Yugoslavian	57	1	41%	49	1	43%	not significant
Indian		–			1		
Polish		2			1		
German		1			1		
Half-caste Australian aborigines		3			–		

percentage of women of British nationality were observed among the abortion series than among the controls (χ^2 [1] 7.802, P = 0.01). This may indicate that British women here had more abortions or that they seek medical attention more readily when a pregnancy threatened to abort than do the non British. As far as the distribution of other nationalities is concerned, there were no obvious differences.

Table III tabulates the results of the various immunologic tests performed as detailed under Materials and Methods. This includes the evaluation of antibodies produced against red cell antigens, γ-globulin and a possible wide variety of disease states, the

latter being determined by the 'C-reactive' protein test (3 4). No statistically significant variations were recorded between the aborters and nonaborters for the occurrence of positive reactions obtained in each of the tests carried out. Of interest, however was the observation that the combined immunologic response between the two series of mothers differed significantly (χ^2 [1] 5.859 P = 0.05)

Table IV shows the recorded distribution and frequency of the Gm serum groups. No significant variations were noted for the distribution of the Gm gene frequencies between the aborters and nonaborters. Their combined frequency was also comparable with the observed findings established for the West Australian population (6) indicating that the aborters and nonaborters examined can be considered as a representative portion of the population. However the phenotypes Gm (a+b+x+) appeared to be significantly higher in aborters than in nonaborters (χ^2 [1] 5.419 P = 0.05) The explanation for this interesting finding is obscure but certainly merits further study

Table V details the results of the haptoglobin, transferrin and Inv(1) serum groups. No significant variation was apparent to suggest that these factors could in any way be associated with the frequent production of anti Tj^a like hemolysins in the aborters.

Discussion and Conclusions

The present investigation shows that no significant differences exist between the haptoglobin, transferrin, Inv(1) and the Gm types of aborters producing anti Tj^a like hemolysins and the nonaborters lacking this factor The Gm(a+b+x+) phenotype variation observed between the two series of mothers cannot, however be explained in the same terms and probably reflects the influence of other factors which have so far not been determined.

The low incidence of haptoglobin deficiency and ahaptoglobinemia is interesting from the point of view of anti-Tj -like hemolysin production although these hemolysins possess the ability to autohemolyze the mother's own red cells *in vitro* (7) an effect *in vivo* has, from clinical observations, only been assumed not to exist. Bars and Lewis (1) were able to establish that ahaptoglobinemia can be associated with intra or extra vascular *in vivo* hemolysis it is thus possible to conclude that a similar situation can be expected for the action of anti Tj^a like hemolysins if this autohemolysin is also active

Table III

The frequency of various positive immunological findings observed between the control and abortion series of mothers examined during pregnancy

Test	Control series: No. examined	Control series: Positive reactions	Abortion series: No. examined	Abortion series: Positive reactions	Chi-square (Yates correction applied)	
Indirect Coombs	204	9	288	21	$\chi^2(1)$ 1.263	not significant
Ficin		23		37	$\chi^2(1)$ 0.149	
Saline		7		6	$\chi^2(1)$ 0.401	
C-reactive protein		14		31	$\chi^2(1)$ 1.743	
SNagg Gm( )		6		14	$\chi^2(1)$ 0.090	
SNagg Gm( )		1		4	$\chi^2(1)$ 0.273	
SNagg Gm(b)		5		11	$\chi^2(1)$ 0.342	
Total immunological response	204	65	288	124	$\chi^2(1)$ 3.859, (P = 0.05)	

The identification of human anti-gamma globulin factors.

Table IV

The frequency of the Gm serum groups observed between aborters, nonaborters and random population (Red Cross donors [6])

	Number tested	Gm phenotypes: Gm (+ -b-)	Gm (+ +b-)	Gm (+ +b+)	Gm (+a-b+)	Gm (a- -b+)	Chi-square	Gene frequencies: Gm	Gm	Gm b	Chi-square
Non-aborters	204	12	19	20	61	92	$\chi^2(4)$ 8.374	0.250	0.100	0.650	$\chi^2(2)$ 5.574
Aborters	288	15	17	51	93	113	not significant	0.232	0.126	0.642	$\chi^2(2)$ 5.232
Total	492	27	36	71	154	203	$\chi^2(4)$ 1.216	0.240	0.115	0.645	$\chi^2(2)$ 3.297
Red Cross donors	300	20	22	37	91	130	not significant	0.251	0.103	0.616	$\chi^2(2)$ 1.484

in vivo The observation that only 2% of the aborters showed ahaptoglobinemia, when at least 80% should have been expected, supports our previous assumption that the anti Tj like hemolysin does probably not cause autohemolysis *in vivo*

In an attempt to reveal other characteristics common to the aborters producing anti-Tj^a-like hemolysins, an extensive search for acquired antibodies was carried out. In this particular study, no significant variations could be established between the ability of control and test groups to produce antibodies to erythrocyte antigens detectable by indirect Coombs ficin, or saline antibody techniques. Similarly the frequency of C-reactive protein and of antibodies reactive with red cells sensitized with specific Gm(a) Gm(b) and Gm(x)γ-globulins did not differ significantly in the test and control groups.

When the various types of positive antibody tests were combined and considered as an index of immunologic response it was of particular interest to note that the difference in this index between the aborters and nonaborters was statistically significant (Table II χ^2([1] 5 859 P = 0 05) However the aborters, as a consequence of frequent loss of early conceptions, appear to receive more direct medical attention in the form of transfusions (red cells, plasma, fibrinogen, γ-globulin etc.) vaccinations, or hormone therapy than do the nonaborters. Hence, this significant variation does not necessarily imply that the aborters are immunologically more responsive in producing various types of antibodies.

These observations do however raise the question as to whether Rh-negative aborters could in fact more frequently be immunized by the Rh factor than the nonaborters. As is evident in Table VI, the small numbers of Rh negative mothers examined among the present series of aborters do appear more often to produce indirect Coombs reacting antibodies than Rh negative nonaborters. Although the difference for this small series is not statistically significant, future studies in this direction could assist in establishing more conclusive reasons why Rh immunized mothers with low Rh antibody titer values experience more abortions than those with higher Rh antibody titer values (8)

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Investigations	Control series Total	Control series Observed	Abortion series Total	Abortion series Observed	Chi-square (Yates correction applied)
Haptoglobin type 1-1		29		39	
Haptoglobin type 2-1	200	94	285	129	$\chi^2(2)$ 0.524 not significant
Haptoglobin type 2-2		77		117	
Haptoglobin deficiency	200	2	285	3	
Ahaptoglobinaemia	204	4	288	3	
Transferrin type CC	204	204	285	283	
CB		0		2	
Inv(1+)	200	164	260	198	$\chi^2(1)$ 1.969 not significant
Inv(1−)		36		62	

Table VI

The frequency of indirect Coombs positive test observed between the Rh negative and Rh positive aborters and nonaborters.

	Degree of indirect Coombs reactivity*	Rh negative mothers I.D.C. test Positive	Rh negative mothers I.D.C. test Negative	Rh positive mothers I.D.C. test Positive	Rh positive mothers I.D.C. test Negative	Rh negative and Rh positive mothers Combined Positive	Rh negative and Rh positive mothers Combined Negative	Mothers
288 aborters	1	1		5		6		
	2	3		4		7		
	3	2		2		4		Rh neg. 48 = 16%
	4	3		1		4		Rh pos. 240 = 81%
Total		9 = 18.7%	39	12 = 5.0%	228	21 = 7.2%	267	
204 nonaborters	1	2		4		6		
	2	1		2		3		
	3					–		Rh neg. 37 = 18%
	4							Rh pos. 167 = 82%
Total		3 = 8.1%	34	6 = 3.5%	161	9 = 4.4%	195	
Chi-square (Yates correction applied)		$\chi^2(1)$ 1.173 not significant		$\chi^2(1)$ 0.188 not significant		$\chi^2(1)$ 1.283 not significant		

* 4 denotes strong agglutination 1 denotes weak agglutination

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Summary

The serum haptoglobin, transferrin, Inv and Gm types have been determined in 288 bortens and 204 nonaborters. No association was noted between the genetic type and the presence or bsence of the anti-Tj^a-like hemolysins frequently found in sera of aborters. The absence of marked differences in the distribution of haptoglobin deficiency and ahaptoglobinemia also confirmed that the established anti-Tj^a-like hemolysins, which are autohemolytic *in vitro*, probably do not produce hemolysins *in vivo*. Production of various antibodies was significantly more frequent in the series of aborters than in nonaborters. It is possible that this difference was influenced by the different mode of clinical ttention the aborters received in the form of transfusions, hormone therapy or vaccinations.

Zusammenfassung

Serum-Haptoglobin, Transferrin, Inv- und Gm-Typen wurden im Serum von 288 Frauen mit Abort und von 204 Frauen ohne Abort bestimmt. Es ergab sich keine Beziehung zwischen dem genetischen Typ und dem Vorkommen oder Fehlen von Anti-Tj^a-Hämolysinen, wie sie im Serum von Frauen mit Abort häufig gefunden werden. Das Fehlen deutlicher Unterschiede in der Verteilung von Haptoglobinmangel und Ahaptoglobinämie bestätigt, daß die Anti-Tj^a-Hämolysine, die *in vitro* autohämolytisch wirken, wahrscheinlich *in vivo* keine Hämolyse hervorrufen. Die Produktion verschiedener Antikörper war bei Frauen mit Abort signifikant häufiger als bei Frauen ohne Abort. Es ist möglich, daß diese Differenz beeinflußt wurde durch eine unterschiedliche klinische Behandlung der Frauen mit Abort in Form von Transfusionen, Hormontherapie und Impfungen.

Résumé

L'haptoglobine, la transferrine, les types Inv et Gm ont été déterminés dans le sérum de 288 femmes ayant eu un avortement et dans le sérum de 204 femmes en ayant pas eu. Aucune relation ne put être démontrée entre le type génétique et la présence ou l'absence d'hémolysines anti-Tj^a telles qu'on les trouve dans le sérum de femmes ayant eu un avortement. Le manque de différences distinctes entre la distribution de la déficience en haptoglobine et de l'haptoglobinémie confirme le fait que les hémolysines anti-Tj^a qui ont un effet hémolytique in vitro ne provoquent probablement pas d'hémolyse in vivo. La production de différents anticorps est de façon significative plus fréquente chez les femmes ayant eu un avortement que chez celles n'en ayant pas eu. Il est possible que cett différence soit due aux soins cliniques différents, tels que transfusions de sang, thérapie hormonale et vaccination, qu'ont reçus les femmes ayant eu des avortements.

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Paraproteinämie und plasmaretikuläre Zellproliferation bei Polycythaemia vera

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Über das Vorkommen von Paraproteinen bei der Polycythaemia vera war bis vor kurzem nur wenig bekannt. Auf dem Deutschen Hämatologentag in Innsbruck wurde 1965 über die erste eigene Beobachtung berichtet (5). Im Dezember des gleichen Jahres stellten Dittmar *et al.* während der 8. Tagung der Amerikanischen Gesellschaft für Hämatologie in Philadelphia einen Patienten mit Polycythaemia vera vor, den sie protein-pathologisch sehr eingehend untersucht hatten (1). Sie konnten im Patientenserum Gamma-G-, Gamma-A- und Mikro-Paraproteine nachweisen sowie im Urin Bence-Jones-Proteine vom K- und L-Typ. Zytomorphologisch sahen sie eine ausgeprägte Infiltration des Knochenmarks mit atypischen Plasmazellen. Eine ähnliche Befundkonstellation wurde vor kurzem in der Schweiz beobachtet (3).

Bis dahin war im Schrifttum nur über 3 Fälle von Polyzythämie berichtet worden, die papierelektrophoretisch einen auffälligen Gradienten hatten erkennen lassen (zit. nach 1). Ein immunologischer Nachweis der Paraproteine bzw. eine eingehendere Charakterisierung war dabei nicht erfolgt. Dagegen ließen entsprechende morphologische Befunde bei Polyzythämie-Patienten schon früher an das gleichzeitige Vorkommen eines Plasmozytoms denken (6).

Im folgenden möchten wir über 3 eigene Fälle von Paraproteinämie bei Polycythaemia vera berichten. Bei allen Patienten war die

Diagnose einer Polycythaemia vera klinisch und zyto-morphologisch zu objektivieren. Sie wurden mit Radio-Phosphor behandelt und ließen nach mehrjährigem Verlauf Gamma A Paraproteine und eine mehr oder weniger ausgeprägte plasmazelluläre bzw. lymphoretikuläre Knochenmarkinfiltration erkennen.

Kasuistik

1. Bei dem jetzt 51 Jahre alten Patienten (J. St., Kr. Bl.-Nr. 13/808/65) wurde erstmals 1962 anläßlich einer profusen Darmblutung ein Milztumor festgestellt und im Herbst 1963 in einem auswärtigen Krankenhaus die Diagnose einer Polyzythämie gesichert. Klinisch zeigte er zu jener Zeit die charakteristische Rubro-Zyanose und eine starke Injektion beider Konjunktiven. Milz und Leber überragten den Rippenbogen um etwa 2 QF. Die übrigen klinischen Befunde waren im wesentlichen unauffällig. RR 205 per 110 mm Hg. BSG ½ mm n. W. Hämoglobin 17,3 g%. Erythrozyten 6,5 Millionen/mm³. Leukozyten 13.600/mm³. Thrombozyten 1,5 Millionen/mm³. Retikulozyten 16‰. Hämatokrit 66%, alkalische Leukozyten-Phosphatase: Index 246. Urin: Eiweiß positiv, Esbach 0,7‰. Seinerzeit wurde eine Serum-Elektrophorese oder eine Bence-Jones-Probe im Urin nicht durchgeführt. Die Behandlung erfolgte ambulant mit Radio-Phosphor (Oktober 1963: 5 mC, Februar 1964: 3 mC, August 1964: 10 mC P32). Nach zunächst zufriedenstellender Besserung der klinischen Symptomatik kam es zu Beginn des Jahres 1965 zu einer erneuten Verschlechterung des Zustandsbildes, so daß der Patient im Februar 1965 stationär in der Medizinischen Universitätsklinik Freiburg im Breisgau aufgenommen wurde. Die klinischen Befunde entsprachen den 1963 auswärts erhobenen.

Die Veränderungen des peripheren Blutes waren weniger ausgeprägt. Urin: Eiweiß positiv, Esbach 0,9‰, Bence-Jones-Probe positiv. Rest-N 44 mg%. Endogene Clearance und Phenolrotprobe zeigten eine deutliche Einschränkung der Nierenfunktion. Papierelektrophoretisch stellte sich im Serum ein schlanker Paraproteingradient im Beta-Bereich dar. Immunelektrophoretisch ließen sich Gamma-A-Paraproteine nachweisen, sedimentationsanalytisch fand sich eine Vermehrung der 7-S-Globuline auf 33 Relativprozente (Abb. 1). Im normal zellreichen Sternalpunktat sah man bei ausgeglichenem erythrogranulopoetischen Verhältnis eine deutliche, z. T. fleckförmige Plasmazellvermehrung. Die Plasmazellen selbst zeigten ein auffälliges, vakuolisiertes Zytoplasma (Abb. 2). Vereinzelt fanden sich großkernige, plasmazellähnliche Elemente mit lockerer Kernstruktur. Das Beckenkammtrepanat zeigte unauffällige Knochenanteile mit mittelweiten, mäßig zellhaltigen Markräumen, eine Vermehrung der roten Vorstufen, stellenweise eher verminderte Megakaryozytenzahlen und ein gehäuftes Vorkommen plasmaretikulärer Zellelemente. Röntgenologisch waren keine osteolytischen Skelettdefekte nachweisbar.

2. Der jetzt 65jährige Patient (A. B., Kr. Bl.-Nr. 7608/59) wurde erstmals 1939 wegen eines Diabetes mellitus in der Medizinischen Klinik Lübeck aufgenommen. Bereits damals wiesen die charakteristischen Veränderungen im peripheren Blut auf eine Polycythaemia vera hin: Hb 21,5 g%. Erythrozyten 7,0 Mill./mm³. Leukozyten 10 400/mm³. Thrombozyten 305.000/mm³. Retikulozyten 5‰. Hämatokrit 64%. Damals wurde nur eine Insulinbehandlung eingeleitet. 1962 zeigten sich die peripheren Blutwerte anläßlich einer stationären Nachuntersuchung nur wenig verändert. Alkalische Leukozyten-Phosphatase: Index 227. Die Sternalpunktion ergab damals ein hyperplastisches Mark mit gesteigerter Erythropoese. Die Behandlung der Polycythaemia vera wurde mit 9 mC P32 eingeleitet. Der Patient wurde in der Folgezeit weitgehend beschwerdefrei. Im August 1965 erkrankte er akut an einer Fußphlegmone mit Staphylokokkensepsis und wurde deshalb wieder stationär aufgenommen. Bei der Aufnahme

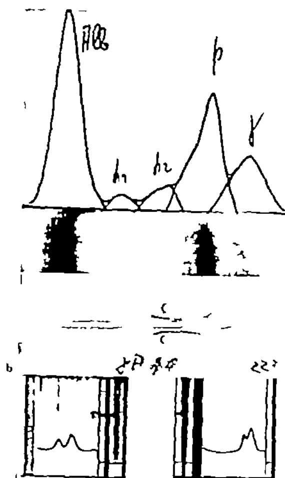


Abb. 1. Serologische Befunde von Fall 1.
(a) Papierpherogramm: Gesamt-Eiweiß = 8,5 g%, Albumin 46 relativ%; Globuline: alpha$_1$ 5, alpha$_2$ 7, beta 27, gamma 15 relativ%. (b) Immunelektrophorese mit spezifischem Anti-Gamma-A-Globulinserum: Deutliche Verstärkung, Deformierung und Spaltung der Präzipitationslinie im Patientenserum (oben). Normalserum als Kontrolle (unten). (c) Ultrazentrifugendiagramm. Aufnahmen 26 und 90 Minuten nach Erreichen der Hochstgeschwindigkeit (59780 UpM). Sedimentationsrichtung von rechts nach links. A = 62, G = 35, M = 3 relativ% (S_{20W} = 17,55 S).

überragten Leber und Milz den Rippenbogen um 2 bzw. 1 QF. Hb 12,4 g%, Erythrozyten 3,9 Mill./mm³, Leukozyten 11400/mm³ mit deutlicher Linksverschiebung im Differential-Ausstrich. BSG 69/110 mm n. W. Auch nach Abklingen des septischen Allgemeininfektes und des lokalen Befundes unter chirurgischer und antibiotischer Behandlung zeigte sich in der Papierelektrophorese ein schlanker Gradient im Gammabereich. Die Bence-Jones-Probe im Patientenurin war negativ. Die im serologischen Laboratorium der Medizinischen Universitätsklinik Freiburg i. Br. vorgenommene Immunelektrophorese ließ Gamma-A-Paraproteine nachweisen. Sedimentationsanalytisch fand sich

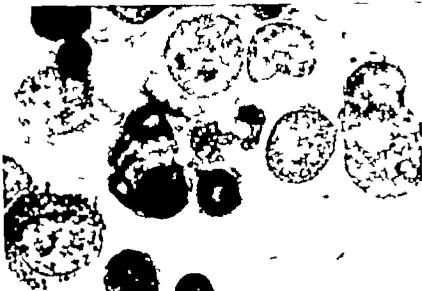


Abb. 2. Zytomorphologischer Befund von Fall 1
Plasmazellen mit auffälligen Plasmavakuolen. Retikulumzellen mit großem, lockeren Kern (Sternalmark, Vergrößerung 1 : 1520)

eine Vermehrung der 7-S-Globuline auf 37% und eine mit 8,70 S wandernde zusätzliche Globulinfraktion. Die physiologischen Makroglobuline fehlten (Abb. 3). Im zellreichen Sternalpunktat sah man außer einer lebhaften Erythropoese eine massive Durchsetzung des Knochenmarks mit überwiegend ausgewachsenen Plasmazellen verschiedener Größe (Abb. 4). Das Beckenkammrepanat zeigte unauffällige knöcherne Anteile und reichlich Zellinseln im Fettmark. Die Zellinseln bestanden zum Teil aus Blutbildungsherden, zum Teil überwiegend oder ausschließlich aus plasmazellulären Elementen. Die Plasmazellen selbst waren ungleich groß, die Kerne leicht polymorph. Insgesamt entsprach das Bild einem Plasmocytom. Röntgenologisch waren auch hier keine osteolytischen Skelettherde nachweisbar.

3. Bei dem jetzt 63jährigen Patienten (F. S., Kr.-Bl. N. 4941/53) wurde bei einer augenärztlichen Untersuchung 1953 eine Polyglobulie festgestellt. Seit 1954 befand er sich unter dem klinischen Bild einer Polycythaemia vera wiederholt in stationärer Beobachtung und Behandlung der Medizinischen Universitäts-Klinik Göttingen. Im Vordergrund standen die durch die Erythrocytenvermehrung verursachten Beschwerden (Hb zwischen 19 und 21%g, Erythrocyten 6–8 Mill./mm³, Hämatokrit 70–80%). Ab 1961 eine Neigung zu Thrombophlebitiden bei eher niedrigen Thrombocytenwerten (101 000 bis 140 000/mm³). Damals fiel auch erstmals eine Lebervergrößerung auf (+2 QF), die Milz war seit 1954 gerade am Rippenbogen tastbar. Die Rechtsverschiebung des Weltmannbandes (8. R.), die pathologische Takata-Reaktion (50 mg% $HgCl_2$) mit leichter Bilirubin-Erhöhung (1,32 mg%) bei noch normaler Bromthaleinretention (0,5% nach 60') wurden als Ausdruck einer gestörten Leberdurchblutung angesehen. Auf die Behandlung mit Radio-Phosphor (Gesamtdosis 24,1 mC P^{32} in 5 Therapiedosen) kam es jeweils zu einer 2–3 Jahre anhaltenden Senkung der Erythrocytenwerte. Im Februar 1966 wurde zur Frage der therapiebedingten Faservermehrung

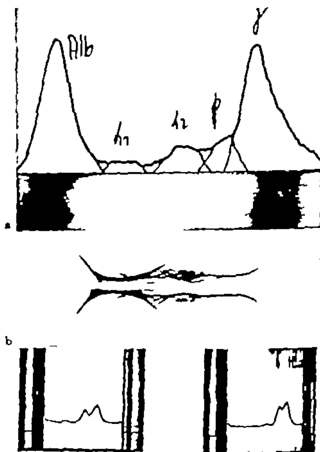


Abb. 3. Serologische Befunde von Fall 2.
(a) Papierpherogramm: Gesamt-Eiweiß = 8,7 g%, Albumin 37 relativ%, Globuline: alpha 1 5, alpha 2 9, beta 7, gamma 42 relativ%. (b) Immunelektrophorese mit polyvalentem Antihumanserum: Verstärkte und erheblich deformierte Gamma-A-Präzipitationslinie im Patientenserum (oben), Normalserum als Kontrolle (unten). (c) Ultrazentrifugendiagramm: Aufnahmen 26 und 70 Minuten nach Erreichen der Höchstgeschwindigkeit (59780 U.p.M.). Sedimentationsrichtung von rechts nach links. A = 60, G = 37, Z = 3 relativ% (S_{20W} = 8,70 S), M = 0 relativ%.

im Knochenmark eine Beckenkamm-Biopsie durchgeführt. Dabei fiel neben einer allgemeinen Steigerung der Zelldichte eine fleckförmige Vermehrung kleiner lymphoider Zellen auf (Abb. 5). Die daraufhin angefertigte Elektrophorese zeigt eine schmalbasige Zacke im Gammabereich. Dem entsprechen in der Immun-Elektrophorese Gamma-A-Paraproteine. Sedimentationsanalytisch fand sich eine Vermehrung der 7-S-Globuline auf 38% (Abb. 6). Weitere Laboruntersuchungen wiesen auf einen daneben bestehenden Leberparenchymschaden hin (GPT 30 IE, alkalische Phosphatase 71 IE, Prothrombin 62%, Bromthaleinretention 15% nach 45'). Röntgenologisch waren keine myelomtypischen Skelettherde nachweisbar.

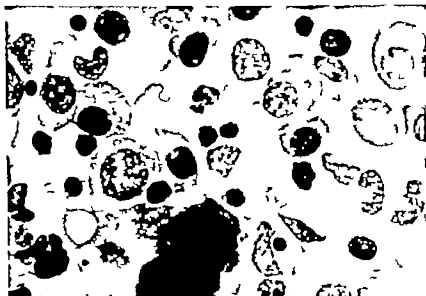


Abb. 4. Zytomorphologischer Befund von Fall 2. Plasmazelluläre Knochenmarks-infiltration, 1 Megakaryozyt (Sternalmark, Vergrößerung 1 : 670)



Abb. 5. Zytomorphologischer Befund von Fall 3. Fleckförmige Vermehrung lymphoider Retikulumzellen im zelldichten Beckenkammtrepanat.

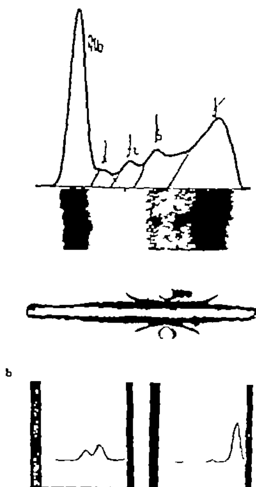


Abb. 6. Serologische Befunde von Fall 3.
(a) Papierpherogramm Gesamt-Eiweiß 7,2 g% Albumine 41 relativ% Globuline alpha$_1$ 3, alpha$_2$ 7 beta 11 gamma 38 relativ%. (b) Immunelektrophorese mit spezifischem Anti-Gamma-A-Globulinserum Deutliche Verstärkung, teilweise Deformierung und bandartige Verschleierung der Präzipitationslinie im Patientenserum. Zusätzlich bandförmige Präzipitation, kathodisch der Auftragsstelle (oben) Normalserum als Kontrolle unten (c) Ultrazentrifugendiagramm Aufnahmen 14 und 74 Minuten nach Erreichen der Hochstgeschwindigkeit (59780 UpM) Sedimentationsrichtung von rechts nach links A = 59, G = 38, M = 3 relativ% S_{20W} = 17,27 S

Besprechung

Das gleichzeitige Vorkommen paraproteinbildender lymphoplasmaretikulärer Zellproliferationen bei verschiedenen Hämoblastosen ist bekannt 9 Beobachtungen der Art, wie sie eingangs

erwähnt und hier ausführlich mitgeteilt wurden, waren somit theoretisch zu erwarten. Ganz besonders ist in diesem Zusammenhang auf das gleichzeitige Vorkommen von Paraproteinen und plasmaretikulärer (2) bzw. lymphoretikulärer (4) Zellproliferationen bei der chronischen Erythroblastose hinzuweisen.

Eine gesicherte Erklärung der Pathogenese dieser Fälle steht noch immer aus. Wir können deshalb für die hier mitgeteilten Beobachtungen nur die grundsätzlich zu erörternden Möglichkeiten nebeneinanderstellen.

1. Könnte es sich um ein zufälliges Zusammentreffen zweier Hämoblastosen – Polycythaemia vera und Plasmozytom – handeln. Bei der relativen Seltenheit der beiden Erkrankungen ist diese Möglichkeit wenig wahrscheinlich.

2. Verschiedene Zellstränge proliferieren auf einen gemeinsamen «Wucherungsreiz» im Sinne Wildhacks (10). Es handelt sich also nicht um eine Kombination zweier Hämoblastosen, sondern um eine pluriblastische Neoplasie. Diese Deutung wäre gerade bei der Polyzythämie besonders einleuchtend, da die Erkrankung ja primär schon mit einer Proliferation mehrerer Zellstränge einhergeht.

3. Es handelt sich um einen Wechsel der Zellformation, wie er bei Hämoblastosen von Rohr (8) beschrieben wurde. An diese Möglichkeit läßt vor allem der Krankheitsverlauf bei dem zweiten, hier mitgeteilten Fall denken, bei dem die morphologischen und klinischen Erscheinungen der Polycythämie zum Zeitpunkt der nachgewiesenen plasmazellulären Proliferationen weitgehend zurückgetreten waren.

4. Plasmazelluläre Proliferation und Paraproteinämie sind ursächlich mit der durchgeführten Radio-Phosphor-Behandlung im Sinne einer strahleninduzierten Neoplasie in Zusammenhang zu bringen. Dabei ist auf die Beobachtungen von Pohl (7) hinzuweisen, der 1960 Plasmozytome bei 3 ärztlichen Hilfspersonen beschrieb, die über das gewöhnliche Maß hinaus Röntgenstrahlen ausgesetzt waren.

Wie bereits erwähnt, läßt es sich nicht mit Sicherheit entscheiden, welche dieser Erklärungen wirklich zutrifft. Wir selbst möchten der zweiten Möglichkeit die größte Bedeutung beimessen und deuten die hier mitgeteilten Beobachtungen im Sinne pluriblastischer Neoplasien.

Anmerkung bei der Korrektur: Während der Drucklegung dieser Arbeit berichten Heinle *et al.* (Arch. Intern. Med. *118*, 331 1966) über einen weiteren Fall von Polycythaemie und Plasmocytom. Die Autoren geben gleichzeitig einen ausführlichen Überblick über die diesbezügliche Literatur

Zusammenfassung

Es wird über 3 Patienten mit klinisch und hämatologisch gesicherter Polycythaemia vera berichtet, die mit Radio-Phosphor behandelt worden waren. Bei späteren Untersuchungen ließ sich eine Gamma-A-Paraproteinämie objektivieren. In einem Fall fand sich zusätzlich eine Bence-Jones-Proteinurie. Sternalpunktion und Beckenkammtrepanation zeigten eine deutliche, zum Teil fleckförmige Vermehrung atypischer Plasmazellen. Osteolytische Skelettherde waren röntgenologisch nicht nachweisbar. Die verschiedenen pathogenetischen Möglichkeiten und Zusammenhänge werden diskutiert.

Summary

In 3 patients with polycythemia vera, who were treated with radiophosphorus, gamma-A-paraproteinemia was diagnosed by immunoelectrophoresis and ultracentrifugal analysis. One of the patients had also Bence Jones proteinuria. The histological respectively cytological examination of a bone marrow biopsy showed spotty infiltration of lymphoid reticulum cells and atypical plasma cells. X-ray examinations did not show punched-out osteolytic areas. The possible pathogenesis of this disease entity is discussed.

Die Untersuchungen erfolgten mit Unterstützung durch das Bundesministerium für Wissenschaftliche Forschung (Kennzeichen St. Sch. 157) und durch die Deutsche Forschungsgemeinschaft.

Résumé

Rapport sur 3 malades atteints de polycythémie vraie traités radio-phosphore. Au cours d'examens ultérieurs, une paraprotéinémie gamma A put être mise en évidence à l'aide d analyses par électrophorèse et ultracentrifuge. Les urines d un des malades contenaient de la proteine de Bence Jones. La ponction sternale et la trépanation de la crête iliaque mirent en evidence une nette augmentation de plasmocytes atypiques, par places en groupes. L examen roentgenologique du skelette ne révéla aucune lésion ostéolytique. Les differentes possibilites de la pathogénie de ces cas sont discutees

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Cellular Changes in the Bone Marrow Following Chronic Treatment of Rats with Cortisol*

J. H. Morrison and J. R. Toepfer

Adrenocortical steroids exert a marked lymphopenic action on the bone marrow of endocrine-deficient animals (1). With few exceptions (2) comparable treatment of normal animals with steroid hormones produces little or no effect on the lymphocyte population of the marrow. Numerous studies have indicated that extensive interchange of lymphocytes may occur between the lymphoid tissues and bone marrow (9). Therefore, it appeared important to investigate the effects of corticosteroids in normal animals under conditions which maintain suppression of lymphoid tissue growth during the experimental period. The present study describe the changes in the marrow cellularity of normal rats treated chronically with doses of cortisol producing rapid and prolonged involution of the thymic tissue.

Materials and Methods

37-day old male Holtzman rats, ranging in weight from 115 to 130 g were used. Littermates were matched by weight into two experimental and three control groups. One experimental group received injections of cortisol (0.05 mg/g rat) at 0, 1, and 2 days (d). These animals were killed on the third day after initiation of cortisol injection. The other experimental group received the same dose of cortisol (0.05 mg/g rat) at 0, 1, 2, 3, and 4 d; these rats were killed on the 5th day. All experimental animals were injected at approximately the same time of day (2 p.m.) to minimize possible diurnal fluctuations in hemopoietic activity. The zero control group were sacrificed at the beginning of the experimental period, the second and third control groups at 3 and 5 d, respectively after the start of the experiments.

All animals were killed at the same time of day (3 p.m.) by a sharp blow applied to the base of the skull. The thymus of each animal was removed, cleared of connective tissue and weighed on a Sartorius balance. The right femurs were then freed of adherent tissue by rubbing with cheese-cloth. The epiphyses of each femur was removed and the

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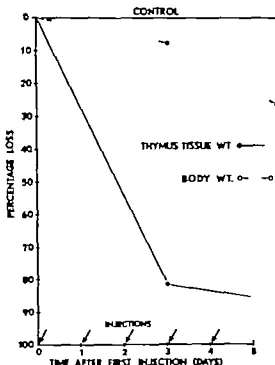


Fig. 1. Per cent loss of thymus tissue weight and body weight of intact rats at 3 and 5 d after initiation of cortisol injections. Each point on the graph represents an average for 6 animals. Arrows indicate days on which intraperitoneal (i.p.) injections of cortisol were given.

remaining bone shaft split lengthwise. The procedure used for the determination of total absolute nucleated cell counts followed the essential steps outlined by GORDON *et al.* (1 Differential cell counts were made on marrow smears stained with Jenner-Giemsa. The morphological criteria used for the identification of the different marrow cells, especially lymphocytes, were those described by HARRIS and BURKE (3 RAMSELL and YOFFEY (5), and MORRISON (4)

Results

The thymi of cortisol-injected animals were markedly in oluted, losing 82 / of their initial weight at 3 d and 89 at 5 d (Fig 1) The animals lost 26 body weight, compared with controls at 5 d after initiation of cortisol injection (Fig 1)

While average total nucleated cell count for the femoral bone marrow of controls was $2.5 \times 10^4/mm^3$ the total marrow cellularity of the injected animals fell belo that of controls at 3 and 5 d, but

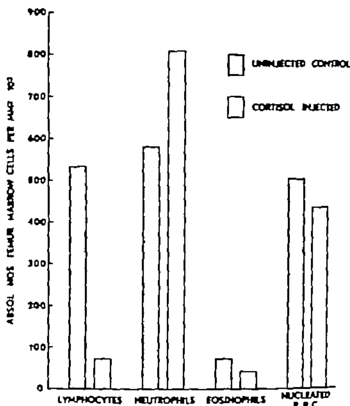


Fig Absolute cell numbers (thousands/mm³) in the femoral bone marrows of rats t 5 days after initiation of cortisol treatment. Bars are means based on 6 rats. Two dots over bar indicates P < 0.01 for values significantly different from uninjected controls.

never reached significance level (P > 0 05) Absolute numbers of bone marrow lymphocytes were significantly reduced (P < 0 01) in both experimental groups (Fig 2) This represented a 70% decrease in the lymphocyte content of the bone marrow at 3 d and 86 at 5 d Transitional cells showed a drop in absolute numbers similar to that of the lymphocytes and reached significance levels (P < 0 01) in both the 3 and 5-day injected groups. A mean drop in absolute numbers of blast cells occurred at three days (P < 0 05) This downward trend in numbers of blast cells was continued in the 5-day injected group where it reached the higher significance level (P < 0 01)

Cells of the neutrophil series showed a progressive increase in absolute numbers during the period of cortisol treatment. This rise in numbers of neutrophils was highly significant (P < 0 01) at 5 d

amounting to a 14 / increase in the concentration of these cells within the bone marrow (Fig 2)

No consistent changes in numbers of eosinophils and nucleated erythroid cells was observed following repeated injections of cortisol (Fig 2) Absolute numbers of primitive reticular cells showed only minor fluctuations in the control and experimental groups. Damaged cells, megakaryocytes, monocytes, plasma cells, and unidentifiable cells were not significantly changed in numbers in the cortisol injected animals.

Discussion

The most striking cellular change in the bone marrow of young rats treated chronically with cortisol is the marked reduction in absolute numbers and percentage of lymphocytes The results of a number of studies indicate that the marrow lymphocytes are hematogenous in origin (2 6, 7 8) If the marrow receives its supply of lymphocytes from extramedullary sources such as the thymus and lymph nodes, one would expect a reduction in numbers of marrow lymphocytes under experimental conditions which resulted in depletion of organized lymphoid tissues (6) The loss of marrow lymphocytes which accompanies reduction in thymic tissue mass of rats treated with cortisol could be interpreted that the thymus is a source of lymphocytes for the marrow

Accumulating evidence indicates that marrow small lymphocytes can enlarge synthesize DNA, and transform to blast cells (8) Our findings of a decrease in numbers of transitional and blast cells coincident with the disappearance of marrow lymphocytes may suggest a developmental relationship between these three forms in the marrow The reduction in numbers of transitional cells and blast forms could be due to the direct inhibitory action of cortisol on the growth and DNA synthesis of marrow lymphocytes, however this question remains unsolved.

There is considerable indirect evidence implicating the lymphocyte as the hemopoietic stem cell in the bone marrow (6, 7 8) It is significant that depletion of the lymphocyte content of the marrow by as much as 86 of control value does not reduce appreciably the level of granulopoiesis and erythropoiesis in the marrows of the cortisol-injected animals. It is possible that only a small part of the total lymphocyte population in the marrow functions as a source for the formation of granulocytes and erythrocytes.

Summary

Chronic treatment of rats with large doses of cortisol resulted in significant reduction of the lymphocyte content of the bone marrow. Cortisol also produced an increase in the absolute numbers of neutrophils in the marrow but did not alter significantly either the nucleated erythroid cells or the total marrow cellularity. The cellular changes in the marrow occurred concomitant with reduction in thymic tissue mass and a loss in total body weight.

Zusammenfassung

Chronische Behandlung von Ratten mit hohen Dosen von Cortisol führte zu einer signifikanten Verminderung der Lymphocyten im Knochenmark. Ferner fand sich eine Zunahme der absoluten Neutrophilenzahl im Knochenmark, jedoch keine signifikante Veränderung der Erythroblasten oder des gesamten Zellgehaltes des Knochenmarkes. Gleichzeitig mit den Veränderungen des Knochenmarkes erfolgten eine Verminderung des Thymusgewebes und eine Abnahme des Körpergewichtes.

Résumé

L'administration à longue durée de fortes doses de cortisol à des rats diminua de façon significative le nombre des lymphocytes dans la moelle osseuse. En plus, le nombre absolu des neutrophiles augmenta dans la moelle osseuse. Par contre, le nombre des érythroblastes et le nombre absolu des cellules de la moelle osseuse ne subit aucun changement significatif. Les changements de la moelle osseuse accompagnèrent en même temps d'une réduction du tissu thymique et d'une perte du poids du corps.

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Comment on the Method of Determining the Trapped Volume of Plasma after Centrifugation Based on the Correlation between the Trapped Volume and the Electrical Conductivity

E. GIBERMAN

A method has been described (1) for determining the trapped volume of plasma after centrifugation The measurement is based on correlation between the trapped volume and the electrical conductivity of the system

The conductivity of a homogeneous isotropic system is proportional to A/L, where L is the distance between the electrodes producing the electric field, and A is the cross sectioned area perpendicular to the field It follows that the conductivity depends on the way the cells are distributed in the system, and not solely on the total volume between the cells. The dominant electrical resistance in a system of resistances coupled in series is the largest one, R_{dL}, if $R_{dL} \gg R$ R_{dL}, where R is the total resistance The average distance between biduscoidal red cells of size ~100 μm^3 is ~100 Å for ~1% trapped volume of plasma, and it is probable that thin layers dL of cells are formed in which the intercellular distance is much smaller (Fig 1)

In the limiting case, in which cells with an average intercellular distance of about 1 Å will form a monolayer which completely blocks thin cross section dL of the system the conductivity will be close to zero (if the conductivity of the cells is neglected) despite the fact that the average trapped volume of the system is far from zero. It is therefore not necessarily permissible to linearly extrapolate the function which correlates conductivity and trapped volume from higher trapped volume values to zero in the region close to trapped volume → 0 (the dotted line in Fig 2)

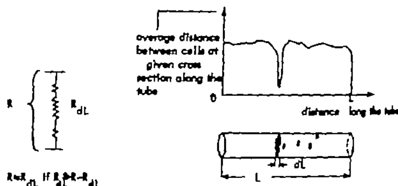


Fig 1



Fig 2

From the previous arguments, it is plausible that the function follows the heavy line indicated in Fig 2, in the region trapped volume → 0 and the trapped volume which should correspond to a given conductivity is t v 2 and not t.v 1 as obtained from the method described (1) which is too low according to the present argument. For experimental systems with $\sqrt{A} \gg L$, the effects on the total conductivity of aggregations of super packed cells is very small, and the linear extrapolation does given a legitimate approximation

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The Infectious Mononucleosis Cell

I. DNA *Synthesis**

H. R. Schumacher, R. B. Moquin, A. E. McFeely and T. K. Maugel

In recent years, emphasis in the study of infectious mononucleosis has shifted from morphologic description of cellular changes to the dynamic relationship of the intracellular metabolism of nucleic acid synthesis (1, 2). Gavosto *et al.* (1) observed that a greater percentage of peripheral mononucleosis leukocytes produced desoxyribonucleic acid (DNA) than normal white blood cells. Epstein and Brecher (3) showed a decline in both DNA and ribonucleic acid (RNA) synthesis by mononucleosis cells as the disease progressed. Galbraith *et al.* (4) demonstrated that the infectious mononucleosis cells have no role in the production of humoral antibody and have many similarities to malignant lymphoid cells.

In this investigation DNA synthesis and mitotic activity of infectious mononucleosis cells were studied by using carbon 14-formate and tritiated thymidine in autoradiographic studies, mitotic counts in peripheral blood and bone marrow, and correlation analysis between guinea pig heterophile titers and nucleic acid synthesis.

Materials and Methods

Patients were selected from the medical services of the York Hospital, York, Pennsylvania. The criteria for acceptance of patients into the study were described previously (5) and relied on three parameters: (1) adequate clinical findings, (2) significant peripheral blood abnormalities, (3) characteristic heterophile agglutination reaction.

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* This investigation was supported in part by Public Health Service Research Grant CA 08245-01 from the National Cancer Institute, and application number 5567 from the American Cancer Society.

Autoradiographic studies were performed at 7 to 10 day intervals on 11 patients with infectious mononucleosis according to the techniques of ADAMIK (6) with some modifications (7). Similar studies were performed on 10 normal individuals on one occasion. The isotopes used were carbon-14-formate with specific activity of 13.4 to 30.5 mC/mM and tritiated thymidine with specific activity of 3.0 C/mM to 8.6 C/mM. Each 4 ml of peripheral blood was exposed to 2.5 μC of isotope for one hour. Slides were prepared with A-R 10 Kodak stripping film and exposed 14 to 21 days before processing. The number of labeled cells and mean grain counts were determined on 1000 leukocytes. Slides were restripped if background contamination was above acceptable limits.

Mitosis was evaluated by counting 1000 nucleated cells from each of 10 normal bone marrow specimens. The mean and standard deviation were calculated. A similar number of cells were counted from each of 7 bone marrow specimens from patients with infectious mononucleosis and compared to the controls. The percentage of infectious mononucleosis cells in the marrow was recorded in each instance. Five hundred leukocytes were observed in the peripheral blood of 17 patients with active disease and the per cent mitoses determined. Similar data were obtained in 10 normal individuals. Cells with double nuclei not manifesting changes compatible with mitosis were counted as non-mitotic cells.

Results

(1) Incorporation of C^{14} formate. The percent of infectious mononucleosis cells labeled was greater during the first 30 days of the illness and gradually declined to control values (0.2 ± 0.2) during the next 20 days. The highest value obtained was 1.4% cells labeled which occurred as early as the ninth day in one case. The average number of grains per labeled cell did not vary significantly with the progression of the disease. The decline in the percentage of the labeled cells was not accompanied by a decrease in the heterophile after guinea pig absorption. There was no relationship between the heterophile and the percent of infectious mononucleosis cells labeled with C^{14} formate ($p > 0.05$).

(2) Incorporation of H^3 thymidine. The percent cells labeled was highest during the first 45 days of the illness. A few values after this time period were observed to be in the normal range (0.2 ± 0.2). The highest value obtained was 6.5 infectious mononucleosis cells labeled on the 6th day of the illness. The lowest number of cells labeled was zero which occurred on the twenty second day. With tritiated thymidine the average number of grains was often in excess of 20 and was much higher than the C^{14} labeled cells. The decline in the percent of infectious mononucleosis cells labeled did not seem graphically to correspond to a decrease in the heterophile after guinea pig absorption, however correlation analysis did demonstrate a relationship ($p < 0.02$). The percent infectious mononucleosis cells labeled with tritiated thymidine and C^{14} formate de-

monstrated a high degree of correlation ($p < 0.01$). With increased observations of tritiated thymidine labeled autoradiographs of infectious mononucleosis cells, cytoplasmic labeling was observed in a paucity of cells. In Fig 1 grains of either cytoplasmic or nuclear origin are shown escaping from a heavily nuclear labeled infectious mononucleosis cell. In Fig 2 grains are definitely present in the cytoplasm.

(3) Unlabeled infectious mononucleosis cells. The percentage of unlabeled infectious mononucleosis cells had a tendency to remain elevated much longer than the labeled group. Some patients maintained over 5% infectious mononucleosis cells 45 days from the onset of their illness.

(4) Mitosis. The mean percentage of infectious mononucleosis cells in the peripheral blood in 17 patients was 58.4%. The mean percent mitosis for 500 cells in the peripheral blood was 0.0. This emphasizes a marked discrepancy between DNA synthesis and mitosis. Ten normal controls counted in a similar manner did not demonstrate mitosis. In Table I the results of bone marrow analysis for mitoses are shown. The number of mitoses in bone marrow from the infectious mononucleosis patients is not significantly different than normal bone marrow ($p > 0.05$).



Fig 1. Extensive nuclear labeling in mononuclear cell with large numbers of grains escaping from the cytoplasm (tritiated thymidine 5.0 C/mM) (× 1000)

Fig 2. Increased labeling in the cytoplasm of mononucleosis cell (tritiated thymidine 5.0 C/mM) (× 1000)

Table I

	No. patients	Percent atypical lymphs	Percent mitoses
Controls	10	—	0.81
Infectious Mononucleosis	7	12.2	1.09

Values expressed as means

Discussion

Carbon[14] formate labels purines and pyrimidines in both DNA and RNA (8). It is incorporated into nucleic acid early in the synthetic process whereas tritiated thymidine reflects only the last steps of DNA production (9). The progressive decline of labeled mononucleosis cells with both isotopes associated with the disappearance of these cells represents the basic limited nature of this benign disease. The correlation between C[14] formate and tritiated thymidine labeled mononucleosis cells suggests that early and late nucleic acid synthesis are related. This apparently holds throughout the entire course of the disease. The relationship between the heterophile antibody titer and the tritiated thymidine labeled mononucleosis cells appeared significant. Evidently as the inciting agent for this limited lymphoproliferative disorder was withdrawn, the stimulus to heterophile antibody synthesis also disappeared and the heteroimmune substance declined. This correlation was not true for C[14] formate and most likely was due to the multiplicity of labeling sites.

The high percentage of labeled cells in DNA synthesis in the peripheral blood of patients with infectious mononucleosis represents cells which should undergo mitosis! This was not in agreement with our data since extremely small numbers of mitotic figures were noted in the peripheral blood. This finding has been observed previously in other laboratories. EPSTEIN and BRECHER (3) postulated four explanations for this discrepancy between DNA synthesis and mitosis (1) The period of DNA synthesis may be very long relative to the period of actual mitosis, (2) the cells may synthesize DNA without ever dividing (3) the DNA synthesis observed may be that of an intracellular virus rather than nuclear DNA (4) the cells may divide only extravascularly possibly because plasma contains some mitosis inhibiting factor or they may circulate only briefly so that

the chance of a mitosis occurring in the peripheral blood becomes negligible. Of the four possibilities, they chose the last explanation However they admitted it was not possible to rule out viral DNA synthesis.

From our present knowledge of infectious mononucleosis and a few assumptions, it is possible to understand why the percentage of infectious mononucleosis cells in mitosis observed in peripheral blood is so low The origin of these cells is most likely lymphatic (10 11) The transit time of the lymphocyte in peripheral blood is probably short (1–3 h) (12) as compared to the granulocyte (9 h) (13) The DNA synthesis time of infectious mononucleosis cells from experimental data would appear to be in the neighborhood of 12 to 30 h (14) If one assumes a mitotic time of 30 min, and means values from the above data, 0 13% of the infectious mononucleosis cells in the peripheral blood should theoretically be in mitosis. This is not in close agreement with our observations, 0 00% or those of EPSTEIN and BRECHER (3) 0 01 / It suggests that the cells are not dividing in the peripheral blood as calculated and, therefore are dividing elsewhere or possibly have an extremely rapid transit time

The bone marrow in this disease is rarely heavily infiltrated with infectious mononucleosis cells The percent mitosis in the bone marrow of these patients was increased slightly but not significantly over normal mean values. The infectious mononucleosis cells in the marrow may be due to peripheral blood contamination However authors believe the latter explanation unlikely in view of the quality of the marrows examined. If the mononucleosis cells are moving from the peripheral blood into the marrow the percent mitoses observed does not indicate increased division in this particular tissue. One possible source of error in this conclusion was the small percentage of mononucleosis cells observed

Earlier investigators suggested that DNA synthesis in the infectious mononucleosis cell is of viral origin (1) This was disproved by autoradiography and microphotometry (15) however virus like particles have been observed in the cytoplasm of a few mononucleosis cells (16) Such location agrees with our autoradiographic findings and suggests the possibility of replicating DNA viruses. The impact of so few cells on total DNA synthesis must be extremely small.

SCHUMACHER, H R Personal Observation.

Summary

Eleven patients with infectious mononucleosis were studied at weekly intervals by means of autoradiography using carbon-14-formate and tritiated thymidine. The cells labeled declined from the onset of the disease and were completely normal by the 45th day. Correlation between C^{14} formate and tritiated thymidine labeling was observed, and discussed from the viewpoint of early and late DNA synthesis. The heterophile antibody titer did correlate with the per cent infectious mononucleosis cells labeled with tritiated thymidine, but not with those labeled with carbon formate. The discrepancy between DNA synthesis and mitoses is discussed in relation to viral DNA synthesis and cellular kinetics.

Zusammenfassung

Bei 11 Patienten mit Mononucleosis infectiosa wurden in wochentlichen Intervallen Untersuchungen mit C^{14} Format und Tritium-markiertem Thymidin vorgenommen. Die markierten Zellen nahmen vom Beginn der Erkrankung ab und waren bis zum 45. Tag vollkommen normal. Es fand sich eine Korrelation zwischen der Beladung mit C^{14} *Format und mit Tritium-markiertem Thymidin. Sie wird im Hinblick auf frühe und* späte DNA-Synthese diskutiert. Der Titer heterophiler Antikörper zeigte eine Korrelation zu den mit Tritium-Thymidin beladenen Drüsenfieberzellen, dagegen nicht zu den mit C^{14} Format beladenen Zellen. Die Diskrepanz zwischen DNA-Synthese und Mitosen wird diskutiert im Hinblick auf eine virale DNA Synthese und auf die Zellkinetik.

Résumé

11 malades atteints de mononucléose infectieuse ont été étudiés à des intervalles hebdomadaires par autoradiographie à l'aide de C^{14}-formate et de thymidine tritiée. Le nombre de cellules radiomarquées diminua dès le début de la maladie et était tout à fait normal le 45e jour. Une relation entre la charge de C^{14}-formate et de thymidine tritiée fut mise en évidence. Elle est discutée du point de vue de la synthèse initiale et tardive de l'ADN. Le taux des anticorps hétérophiles est en relation avec le pourcentage de cellules de Pfeiffer chargées de thymidine tritiée, mais pas avec les cellules marquées au C^{14}-formate. Le paradoxe existant entre la vitesse de synthèse de l'ADN et le nombre de mitoses est commenté en relation avec l'origine virale de l'ADN et la cinétique cellulaire.

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Die intrazelluläre Verteilung von embryonalem Hämoglobin in roten Blutzellen menschlicher Embryonen*

Ein Beitrag zur Ontogenese menschlicher Hämoglobine

E. F. Kleihauer, T. E. Tang und K. Betke

In der menschlichen Ontogenese treten mit dem Wechsel der verschiedenen Blutbildungsperioden (mesoblastische, hepatische und medulläre) nicht nur morphologisch differente Erythrozyten (14) sondern auch drei verschiedene Hämoglobintypen auf, die sich in Struktur und Eigenschaften erheblich unterscheiden. Während wir beim Neugeborenen noch etwa 60% fetales Hämoglobin (HbF $\alpha_2\gamma_2$) finden, ist dieses beim 6 Monate alten Säugling weitgehend durch adulten Blutfarbstoff (HbA, $\alpha_2\beta_2$) ersetzt. In der Embryonalzeit wird neben HbF und einer geringen Menge von HbA außerdem noch embryonales Hämoglobin gebildet, dessen Existenz heute als gesichert gelten kann (6, 8, 12). Dieser Blutfarbstoff wird in Form von zwei Typen, dem Hb Gower 1 und Hb Gower 2 regelmäßig bei Früchten bis zu einer Kopf-Steißlänge von 8,5 cm gefunden. Hb Gower 2 hat die Kettenformel $\alpha_2\varepsilon_2$. Hb Gower 1 besteht wahrscheinlich nur aus ε-Ketten (ε_4). Die Produktion von Hb Gower 1 wird in der Embryonalentwicklung früher eingestellt als die von Hb Gower 2 (6). Das gilt nicht nur für den Menschen, sondern auch für einige Säugetiere (13).

Aus der zeitlich engen Korrelation zwischen den einzelnen Blutbildungsperioden und den verschiedenen Hämoglobintypen läßt sich nun aber keineswegs eine organspezifische Gebundenheit der Blutfarbstoffsynthese ableiten. Dies konnte für das adulte und fetale Hämoglobulin mit der Elutionsmethode zur Darstellung von HbA und HbF in Einzelerythrozyten nachgewiesen werden (2, 3).

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In der vorliegenden Arbeit möchten wir über eine Methode berichten, die es erlaubt, auch embryonales Hämoglobin in roten Blutzellen fixierter Ausstriche zu differenzieren. Damit wird es erstmals möglich, die gesamte Ontogenese der menschlichen Hämoglobine vom embryonalen bis zum adulten Blutfarbstoff zytologisch zu erfassen und die Beziehungen zwischen Morphologie und Hämoglobinsynthese näher zu analysieren.

Material und Methode

Durch Zusammenarbeit mit verschiedenen Frauenkliniken und geburtshilflichen Abteilungen* war es uns möglich, die Untersuchungen an frisch abortierten Embryonen und Foeten mit einer Kopf-Steiß-Länge zwischen 2,4 und 15,0 cm durchzuführen. Das Blut für die Ausstriche wurde durch Punktion des Herzens und der großen Gefäße mit einer fein ausgezogenen Glaskapillare gewonnen. Zur Herstellung von Hämolysaten für die *Hämoglobinelektrophorese* wurde außerdem die Leber in 1,4%iger Kochsalzlösung durch kräftiges Schütteln zerkleinert. Die groben Partikel konnten durch Gazefiltration und spontane Sedimentation entfernt werden. Die Zellen wurden dann fünf mal mit 1,4%iger Kochsalzlösung gewaschen, anschließend mit Aqua dest. oder durch Einfrieren und Auftauen hämolysiert, mit Tetrachlorkohlenstoff ausgeschüttelt und scharf zentrifugiert. Die Elektrophorese erfolgte im Stärkegel mit Tris-EDTA Puffer pH 9,0, Färbung mit Benzidin und Amidoschwarz.

Für die Darstellung von *embryonalem Hämoglobin* in intakten roten Blutzellen haben sich nach zahlreichen Vorversuchen folgende zwei Verfahren am besten bewährt: Das durch Herzpunktion gewonnene Blut der Embryonen wird bei beiden Methoden zunächst mit gleichen Teilen 1%igem Brillantkresylblau in Ringerlösung für 20 Minuten in der feuchten Kammer inkubiert. Die Notwendigkeit dieser Maßnahme wird weiter unten diskutiert.

1. Modifikation. Aus dem inkubierten Blut werden Ausstriche angefertigt, mindestens 2 Stunden an der Luft getrocknet, darauf 5 Minuten in 96%igem vergälltem Äthanol fixiert, gut mit Wasser abgespült und wiederum an der Luft getrocknet. Als Elutionslösung dient der Zitronensäure-Phosphatpuffer nach McIlvaine, pH 3,4 bis 3,5, der im Wasserbad auf 37 °C erwärmt wird. Die fixierten Brillantkresylblaupräparate werden dann in diesem Puffer für 3 Minuten eluiert, anschließend unter fließendem Wasser abgespült und 3 Minuten in Hämatoxylin nach Ehrlich und 3 Minuten in einer 0,1%igen wäßrigen Erythrosinlösung gefärbt.

2. Modifikation. Die Brillantkresylblaupräparate werden an der Luft getrocknet und in 80%igem Äthanol fixiert. Als Elutionslösung wird in diesem Fall der McIlvaine-Puffer mit einem pH von 2,9 bis 2,95 verwendet. Alles weitere entspricht dem Vorgehen wie bei der 1. Modifikation.

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Resultat. Unter diesen Bedingungen wird fetales und adultes Hämoglobin aus den roten Blutzellen herausgelöst, während der embryonale Blutfarbstoff nicht in Lösung geht und durch Anfärben mit Erythrosin sichtbar gemacht werden kann. Gleichzeitig kommt in den völlig eluierten Erythrozyten (leere Schatten) das Retikulozytennetz bzw. der Kern deutlich zur Darstellung (Abb. 2).

Fehler- und Korrekturmöglichkeiten. Die Eluierbarkeit der Hämoglobine wird entscheidend durch pH-Änderungen in der Elutionslösung und die Art der Fixation der Präparate beeinflußt. Die Fixation in 80%igem Äthanol führt regelmäßig zu Vakuolenbildungen in den Megaloblasten und in den Megalozyten, wodurch die zytologische Beurteilung erheblich erschwert wird. Das Entstehen der Vakuolen kann durch Verwendung eines 96%igen Äthanols (Modifikation 1) reduziert werden.

Für die Auswertung und Beurteilung des Elutionsergebnisses sind nur solche Bereiche in den Ausstrichen zu verwerten, in denen die roten Blutzellen morphologisch einwandfrei intakt sind, was durch mikroskopische Kontrolle der Präparate vor der Elution gesichert werden muß. Es empfiehlt sich außerdem im selben Arbeitsgang eine Nabelschnurblutprobe in gleicher Weise zu behandeln, die als Kontrolle für die Eluierbarkeit des fetalen Hämoglobins dient. Wird HbF unter den oben angegebenen Bedingungen nicht vollständig aus den Erythrozyten des Nabelschnurblutes herausgelöst, so muß das pH der Elutionslösung in 0,05 pH-Stufen entsprechend wieder geändert werden.

Ergebnisse

Elektrophoretisch konnten wir bei allen untersuchten Embryonen bis zu einer Kopf-Steißlänge von 8,5 cm die beiden embryonalen Hämoglobinfraktionen Hb Gower 1 und Hb Gower 2 nachweisen (Abb. 1), wobei Hb Gower 1 regelmäßig in geringerer Konzentration als Hb Gower 2 vorhanden war. Der Anteil an adultem Hämoglobin schwankte bei gleich alten Embryonen in Abhängigkeit von der Präparation recht erheblich.

Morphologisch wurden in May-Grünwald-Giemsa-Präparaten bei Embryonen mit einer Kopf-Steißlänge bis einschließlich 10 cm die für die mesoblastische Blutbildungsperiode typischen Megaloblasten und Megalozyten gefunden. Die Verteilung dieser Zellen in den Ausstrichpräparaten war ungleichmäßig mit einer deutlichen Anreicherung an den Randpartien und den Ausziehungen am Ende der Präparate.

Nach der *Elution* findet man unter den angegebenen Bedingungen in Blutausstrichen von Embryonen zahlreiche Megaloblasten, die noch voll mit Hämoglobin gefüllt sind, während andere den Blutfarbstoff teilweise (Intermediärzellen) oder vollständig abgegeben haben (Abb. 2). Daneben sieht man aber auch immer wieder einige kernlose Erythrozyten, die nur teilweise oder überhaupt nicht eluiert sind, ein Befund, den wir in Kontrollproben aus Nabelschnurblut und Blut von Früchten über 10,5 cm Länge

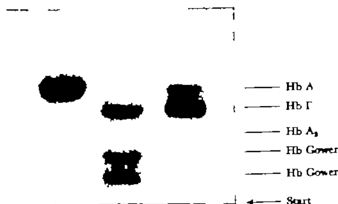


Abb. 1 Stärkegelelektrophorese menschlicher Hämoglobine. Links adultes Hämoglobin (HbA_1 und HbA_2). Mitte Hämolysat von einem 2,5 cm langen Embryo (HbF, Hb Gower 1 und Hb Gower 2). Rechts Nabelschnurhämolysat (HbA und HbF)

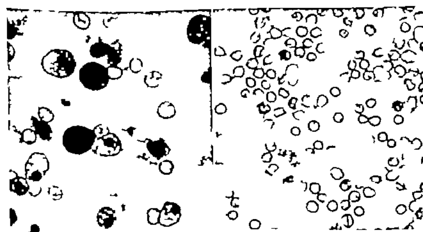


Abb. 2 Eluierte Blutausstriche. Links 3,5 cm langer Embryo. Rechts Nabelschnurblut, in dem Retikulozyten zur Darstellung kommen. Elution nach Modifikation 1

niemals erhoben haben. Eine quantitative Auswertung der Präparate ist recht schwierig, weil die Elutionsergebnisse an verschiedenen Stellen des gleichen Präparates unterschiedlich sein können. Dies ist im wesentlichen darauf zurückzuführen, daß die Blutzellen schon vor der Elution nicht mehr völlig intakt waren. Bei älteren Embryonen ist die geschätzte Zahl der eluierten Megaloblasten größer, während die Intermediärzellen mit dem Entwicklungsalter der Frucht quantitativ nicht zunehmen.

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Haemolysing Properties of Some Exogenous Materials

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Increasing interest has been paid in recent years to the inter action between foreign surfaces and the blood (1—11) since fundamental processes are involved here which must be taken into consideration if the use of artificial organs is not to be limited. In the present paper we have devoted our attention to the damage caused to the blood by certain materials used in medical technology While the increase in plasma haemoglobin reflects only one small aspect of the possible traumatic effect on the blood, it is neverthe less the measurement most frequently cited in the literature. It is also generally understood, simple, relatively exact and reproducible. Our work is based on similar studies published within the last few years (1 2 4 5, 7) and is designed to compare new plastic substances suitable for handling blood with a few wellknown materials.

Methods

The following materials were obtained in the shape of test tubes (internal diameter 13 mm, internal length 97 mm, base radius 6.5 mm) and subjected to several series of tests.

Existing test tubes of glass or polypropylene were coated on the inside with

Silicone oil (AK 500, supplied by Dr A. Wacker, Gesellschaft für elektrochemische Industrie GmbH, Prinzregentenstraße 22, Munich/Germany) in dilution of 1 10 in toluol, applied to glass, then washed and heated to 180 °C for 2 h. *Silastic 382 Medical Grade Elastomer* and *Silastic S-5392* (Dow Corning Corporation, Medical Product Division, Midland, Michigan/USA) diluted with chloroform before adding the catalyst M (stannous octoate), dried for few hours at room temperature and then heated to 100 °C. As the layer tends to separate at the top edge after while, particularly on glass, new tubes must be prepared after every two or three tests. *Araldite Household Adhesive* (Ciba Ltd., Basle, Switzerland) painted on to test tube surface and polymerised t about 100 °C. *Araldite Moulding Resin E* (Ciba Ltd., Basle, Switzerland) same process as with Household Adhesive, using hardening agent HY 951 *Preparation 11073/71* (Ciba Ltd., Basle, Switzerland) approx. 1% solution in cyclohexanone, solvent evaporated in vacuum, dried on at 50 °C. Then Liquemine for 3 min. (Hoffmann-La Roche Ltd., Basle, Switzerland) diluted 1 10 in physiological saline.

The following are materials which were turned on lathe to the same internal measurements and highly polished

Kel-F (polytrifluorochloroethylene) (Pechiney-Saint Gobain, 16, av Matignon, Paris 8e France) *Stainless steel* (18/8/2.5) (Mannesmann Works, Düsseldorf, Germany). *Plexiglas* (metacrylate) (Böhm & Haas, Darmstadt, Germany) *Teflon* (polytetra-fluoroethylene) (Montecatini, Italy) *Araldite Moulding Resin E* (Ciba Ltd., Basle, Switzerland).

Polycarbonate (Montecatini, Italy) (produced by Semadeni Ltd., Galgenfeldweg 8, Berne, Switzerland) and *Polypropylene* (same firm) were purchased directly as test tubes.

The following are lengths of tubing of the same internal diameter and length as our test tubes, but with one end sealed or stuck up

Medical Silastic (601-601) (Dow Corning Corporation, Medical Product Division, Midland, Michigan, USA), with one end stuck up with B–D Vivodil (Medical Adhesive, silicone type A, Dow Corning Corp.) *Portex* (polyvinylchloride) (Portland Plastics Ltd., Hythe, Kent, England)

Graphite-benzalkonium chloride-heparin (GBH) surface (GOTT 1963): Dag 35 and Dag 154 used (graphite in alcohol, 20% solids) (Acheson Colloids Comp., Port Huron, Michigan, USA) diluted with xylol 1 4 and 1:3 respectively covering PVC test tubes (Portex) dried at 100 °C, 3 min in 0 1% zephirol solution (Bayer Leverkusen, Germany), rinsed out with distilled water 3 min in 'Liquemine (Hoffmann-La Roche Ltd Basle, Switzerland) diluted 1 10 in physiological saline, rinsed once with distilled water and dried.

Finally we list those substances tested in form and measurements not exactly corresponding to the dimensions of our test tubes

Latex (Surgeons Gloves) (Seamless Rubber Company New Haven, Connecticut, USA) the middle finger of a size 8½ glove suspended in test tube and attached on the outside with leukoplast (internal measurements 25 × 70 mm). *Polyethylene* (PE 450 PHF) (Clay Adams Incorporated, 141 East 25th Street, New York, USA): piece of tubing 12 mm internal diameter and 90 mm long, sealed t one end. *Polyethylene* (Braun, Melsungen, Germany) similarly sealed, internal diameter 12 mm, length 90 mm.

Before beginning an experiment, the tubes were thoroughly washed out, kept in Fibrinex 10% (A. Lugenbühl Ltd., Könizstrasse 3, Berne Switzerland) at 40—45 °C for 15 min, then rinsed for 3 h and dried at room temperature. With the exception of the first block of tests and of siliconized glass, which we adopted as a sort of standard material for purpose of comparison in our tests, we did not subject the materials to autoclaving, as not all of them are heatresistant. Care was paid, however to maintain identical conditions of treatment and preparation of all the tubes in each series.

For the tests themselves, venous blood was taken from fasting female subjects (nursing staff or patients from the hospital who were receiving no infusions or special therapy) using 120 × 30 gauge needle and allowing the blood to flow freely into a siliconized, graduated round-bottomed flask (60 ml blood/3.0 ml physiological saline per 150 u. heparine) The blood was immediately allowed to stand for 30 min in water bath t 20—25 C (room temperature) in order to ensure comparable temperature conditions for all samples. Next 3.5 ml blood were placed in each test tube with sterile siliconized pipette. The tubes were then closed with stopper covered with polyethylene foil [in the first test block Silastic foil (Dow Corning) was used]. In the first block, the air was first replaced by steam-saturated oxygen with 4% CO_2. The samples were then placed horizontally in non-vibrating shaker (horizontal movement 33 mm, frequency 160/min) and subjected during 3 h to regular smooth and gentle motion at room temperature In order to obtain an initial plasma haemoglobin level for each sample of blood, 3.5 ml were immediately centrifuged (twice 10 min at 1200 g) pipetted off, mixed with cyanide solution and kept at 4 °C until the end of the experiment. The blood of the 8 tubes tested together (using blood from the same subject) was poured into siliconized

centrifuge tubes after shaking, centrifuged at the same time in the same machine pipetted off, mixed with cyanide solution and kept at 4 °C until the haemoglobin determination (maximum 4 h after the test).

The plasma haemoglobin was determined according to the cyanmethaemoglobin method (17) To 2.5 ml cyanide solution [20 g/100 ml distilled water (Dade)] were added 0.5 ml serum. The values were read at 540 µm and 680 µm (average of two determinations for each sample) The values reported in this paper refer to the difference in the haemoglobin content in mg/100 ml plasma (increase in haemolysis) between the initial control value and that obtained after 3 hours shaking in the respective tube.

Statistical evaluation of the results* was carried out by two-way analysis of variance and the F-test. To confirm the plasma haemoglobin determination the χ^2-test was also used.

Results

In order to estimate the mean square error of our haemoglobin determination method, a blood sample was placed in 8 siliconized centrifuge tubes at the same time under the same conditions (3.5 ml per tube) (the whole process being repeated 8 times with blood from different subjects) immediately centrifuged and the plasma haemoglobin determined. The two-way analysis of variance showed that the 8 haemoglobin determinations made at the same time on samples of blood from the same subject did not differ significantly as was also confirmed by the χ^2 test. On the basis of this investigation, our method may be considered sufficiently reproducible. On the other hand, the F test showed that the plasma haemoglobin values of the individual subjects differed significantly from one person to the other This means, that materials can only be compared statistically with other materials which have been tested at the same time and under identical conditions, with the same blood sample (Table I)

We carried out a total of 57 different tests with 8 test tubes each time. The mean total haemoglobin level was at 12.6 g/100 ml, the mean haematocrit level at 39 vol. % As our centrifuge could only take 8 samples at once, we had to vary the selection of materials to be tested. The order in which they were tested depended on the degree of interest (testing of plastic materials, some of which are not reported here, facility and use of the material, etc.) and on our previously available results (good materials – definitely poor materials) For statistical analysis we formed 3 complete blocks of tests, each of which had been performed under identical conditions.

* We should like to thank Dr P Scamen of the Federal Institute for Forestry Research, Birmensdorf, Zurich, Switzerland, most sincerely for his friendly advice and the statistical evaluation of our results.

Table I

Mean square error of a single plasma haemoglobin determination.
Eight simultaneous determinations in 8 different female subjects with total haemoglobin levels between 10.6 and 12.8 g/100 ml and haematocrit levels between 34 and 41 vol.%

Test No.	1	2	3	4	5	6	7	8
$\bar{X}_{n=8}$ in mg/100 ml	20	12	19	18	27	26	19	27
σ exp.	±3	±2	±4	±3	±5	±2	±1	±3
χ^2-test confidence limits	0.8–0.7	0.98–0.9	0.8–0.7	0.7	0.6–0.5	>0.98	>0.98	= 0.9
Two-way analysis of variance,	The values within the test groups are not significantly different at the p = 25% level.							
F-test	The difference between values of different tests is significant (p = 0.1%)							

$\bar{X}_{n=8}$ Mean of 8 single determinations with the same blood sample (mg/100 ml plasma)

p Probability of error

σ exp. $\sqrt{\frac{\Sigma(x-\bar{X})}{n-1}}$ = mean square error of a single determination

χ^2: $\frac{\Sigma(x-\bar{X})}{\bar{X}}$

Table II shows the average increase in plasma haemoglobin content in the three test blocks. The values of the individual tests of a given material within the blocks show significant differences in the two-way test (p = 0.1%). The mean values reported do not represent a normal distribution and therefore it is not possible to calculate a standard deviation. The differences between the different tubes in blocks 1 and 2 are not significant, whereas there are significant differences between those in block 3. These are probably due to the Portex PVC tubing and the two forms of graphite-benzalkonium chloride-heparin treatment, which always produced strikingly high rates of haemolysis (see also Table III).

The maximum values presented in Table II indicate the reliability of a material. Generally speaking they correspond quite well with the means. Nevertheless, siliconized glass, Araldite Household Adhesive, Teflon and the two forms of Silastic, 382 MGE and S-5392, appear to be subject to unpredictable variations, which reduce the value of these tubes for biological purposes. Silastic tubing (Medical Silastic 601-601) and Plexiglas on the

Table II

Test tube material	Complete blocks of tests 1 $\bar{x}$	x max	2 $\bar{x}$	x max	3 $\bar{x}$	x max
	(n = 10)		(n = 9)		(= 5)	
Stainless steel	25	56				
Kel-F	25	52	25	68		
Teflon			26	83		
Plexiglas			19	47	16	38
Polycarbonate	23	56				
Polypropylene	25	59				
Araldite Moulding Resin E	23	52	17*	65		
Araldite Household Adhesive			23	74	26	63
Siliconized glass	23	47	29	117	42	68
Silastic 382 MOE	29	63	21	86	20	29
Silastic S-5392			26	81	16	30
Medical silastic	16	27				
Portex (PVC)					260	508
Dag 154-B-H					500	900
Dag 35-B-H					116	236
F-test Difference	(p = 10%) not signif.		(p = 25%) not signif.		(p = 0.1%) significant	

$\bar{x}$ Arithmetic mean of increase in plasma haemoglobin (mg/100 ml) within the test blocks.
Number of experiments in each block.
Test tube turned from solid material and polished on inside.
Coated glass test tube.

other hand are remarkable for the considerably narrower limits within they vary

From the materials tested in more than one block, it is shown clearly that the difference in the procedure between the first test block (sterile and replacement of air by mixture of steam-saturated oxygen with 4% CO_2) and blocks 2 and 3 had no influence on haemolysis.

In order to present the results obtained from a large number of different blood samples, we have drawn up a list of the average haemolysis values from all the tests carried out with each material (Table III) with the exception of those tests in which the initial haemolysis value was greater than 45 mg/100 ml. In addition we have included in Table III the results obtained with some test tubes

Table III

Test tube material	$\bar{X}$	d	n
Polycarbonate	12	62	34
Medical silastic	14	41	19
Stainless steel	15	68	28
Silastic S-5392	16	82	27
Silastic 382 MGE	18	67	37
Araldite Moulding Resin B	19	63	35
Araldite Household Adhesive	19	73	32
Kel-F	22	85	32
Plexiglas	22	45	16
Polypropylene	23	61	18
Preparation 11073 TI	26	43	11
Teflon	27	77	14
Siliconized glass	39	159	35
Dag 35-B-H	104	216	19
Portex (PVC)	230	625	20
Dag 154-B-H	341	892	17
Polyethylene PE 450 PHF	10	43	5
Polyethylene	16	23	10
Latex	18	25	4

$\bar{X}$ Average plasma haemoglobin increase (mg/100 ml).
n Number of tests
d Difference between maximum and minimum haemolysis to show range. These figures can only be roughly compared with each other as they are not from identical test conditions.

of somewhat different dimensions. These values can only be compared roughly with those of the other experiments. It can be seen however that none of these materials (latex, polyethylene) produces obvious damage to the blood as does Portex tubing for instance. Therefore, significant differences compared with the other good materials are not to be expected here either.

Discussion

Of the 18 exogenous materials we tested, only Portex PVC tubing and the graphite-benzalkonium chloride-heparine surface treatment produced significant haemolysis. The result was supported by the fact, that within the complete blocks of tests, the sequence of the degree of haemolysis for the different materials varied from test to test and seemed to be completely coincidental. We did not correct

our haemoglobin values to a uniform haematocrit level like AEPLI (1) and FREI (7) for instance, as we were unable to discern any correlation between the increase in haemoglobin during our tests and the blood haematocrit.

The significant degree of damage to the blood from graphite benzalkonium chloride-heparine coating of PVC has already been reported in other connection also by INDEGLIA (9). Continued search for other non-thrombogenic and less haemolyzing surfaces, as we tried with the Preparation 11073-T1 appears necessary (6, 10 11). BERGSTROEM and NORDLUND (2) were unable to distinguish any difference between Portex and Tygon tubing in their *in vitro* experiments. Among the materials tested by AEPLI (1) polyvinyl and siliconized glass were found to cause least haemolysis in contrast to the obviously poorer values for Teflon. These differences to our results are probably due, apart from the different experimental conditions to the fact, that partially products of different manufacturers were used. Otherwise our results agree with those of AEPPLI (1) FREI (7) DUC (4) and FLEISCH and FREI (5) in so far as the same materials are involved.

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Summary

Of 18 materials tested, Portex tubing and graphite-benzalkonium chloride-heparine coating were shown to possess significantly more marked haemolysing properties than Plexiglas (metacrylate) Silastic (Dow Corning) Araldite Moulding Resin E (Ciba) and other substances. Comparing these results with those of other authors, it is apparent, that not only the chemical structure but also the origin (manufacturer process) is decisive for the amount of damage a given substance may inflict on the blood.

Zusammenfassung

Es wird eine einfache Versuchsanordnung beschrieben zur Prüfung von körperfremden Materialien, die bei der Konstruktion künstlicher Organe Anwendung finden. Die Testung von 18 verschiedenen Materialproben ergab signifikant starke hämolysierende Eigenschaften einerseits des Portex-Schlauches (PVC) andererseits der Graphit-Benzalkoniumchlorid-Heparin-Beschichtungsmethode gegenüber zum Beispiel Plexiglas (Metacrylat), Silastic (Dow Corning) Araldit Giessharz E (Ciba) u.a.m. Aus dem Vergleich mit den Arbeiten anderer Autoren geht hervor daß nicht nur die chemische Struktur sondern auch die Herkunft (Fabrikationsprozeß, Herstellerfirma) von ausschlaggebender Bedeutung für den Grad der Bluttraumatisierung sein kann.

Résumé

Une méthode simple servant à analyser certaines matières employées dans la construction d'organes artificiels est décrite. L'examen de 18 différentes matières démontre d'une façon significative les propriétés hémolysantes du tube Portex d'une part, et du revêtement intérieur au graphite-benzalkonium chloride-héparine d'autre part, en comparaison p.e. de plexiglas (métacrylate), Silastic (Dow Corning), la résine coulée Araldit (Ciba) et d'autres encore. Il ressort de la comparaison de nos résultats avec ceux d'autres auteurs, que le degré des dommages causés au sang ne tient non seulement à la structure chimique des matières employées mais aussi à leur origine (méthode de fabrication, firme) qui peut être d'une importance primordiale.

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Medizinische Universitätsklinik, Innsbruck (Vorstand: Prof. Dr. H. Braunsteiner)

Zytochemische Untersuchungen zur Entwicklung der grossen mononukleären Zellen des Hautfensters

F. Schmalzl und H. Braunsteiner

Die Mitteilung Rebucks über die Untersuchung ausgewanderter Zellen am Hautfenster löste eine lebhafte Diskussion über die bei dieser Methode auftretenden mononukleären Zellen aus. In zahlreichen Arbeiten wurde versucht, eine Abklärung der Herkunft und des funktionellen Charakters dieser Zellen zu erreichen. Auch zytochemische Untersuchungsmethoden wurden herangezogen.

Bezüglich der Herkunft dieser Zellen vertraten Rebuck *et al.* (17, 18) die Ansicht, daß sie sich von aus dem Blut ausgewanderten Lymphozyten zu Makrophagen entwickeln. Andere Autoren leiteten die Mononukleären von ausgewanderten Blutmonozyten ab. Einer dritten Theorie zufolge sollten sie von ausgewanderten Zellen des Unterhautbindegewebes abstammen (Lit. bei 11).

Hinsichtlich der Fermentausstattung dieser Zellen wurde auf den beachtlichen Gehalt an hydrolytischen und oxydativen Fermenten hingewiesen. Wulff (21, 22, 24) führte ausführliche Untersuchungen der Enzyme des oxydativen Stoffwechsels durch und gelangte zum Ergebnis, daß es bei der Entwicklung der Mononukleären zu einer deutlichen Zunahme der Fermente des Tricarbonsäurezyklus und der Atmungskette kommt. Die Transformation wird somit von einer deutlichen Steigerung des aeroben Stoffwechsels in den Mononukleären begleitet. Unterstützt wird diese Auffassung durch kombinierte biochemische und elektronenoptische Untersuchungen (1, 7), bei denen unter anderem in stimulierten Makrophagen des Peritoneums und der Gewebekultur eine Vermehrung und Vergrößerung der Mitochondrien festgestellt

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Diese Untersuchungen wurden mit Unterstützung des Fonds »Kampf dem Krebs« durchgeführt.

wurde. Bezüglich der Zunahme hydrolytischer Fermente in den Mononukleären während der Transformation liegen unterschiedliche Angaben vor. Einzelne Autoren fanden eine Zunahme bestimmter Enzyme, von anderen konnte dies nicht bestätigt werden.

Die vorliegenden Untersuchungen galten deshalb der Frage, ob sich aus der zytochemischen Untersuchung Hinweise auf die Herkunft der Mononukleären ableiten lassen und inwiefern in diesen Zellen parallel zur morphologischen Transformation, zur Umstellung des Energiestoffwechsels und zur gesteigerten Phagozytoseaktivität auch eine Änderung der hydrolytischen Fermentausstattung erfolgt.

Material und Methodik

An *hämatologisch gesunden Probanden* wurden *Hautexkorationen* gesetzt, auf die mindestens 6 Deckgläschen gleichzeitig aufgelegt und in verschiedenen Zeitabständen gewechselt wurden. Die Gesamtdauer der einzelnen Hautfensterversuche lag zwischen 40 und 120 h. Insgesamt wurden 11 Untersuchungsserien durchgeführt, an denen jeweils mehrere ferment- bzw. binnenzytochemische Nachweisverfahren zur Anwendung gelangten. Zu Vergleichszwecken wurden die in normalen Blutzellen feststellbaren Enzymaktivitäten herangezogen.

Naphthol-AS-Acetat-Esterase. Den Nachweis der N-AS-Acetat-Esterase führten wir auf Grund der von LÖFFLER angegebenen Methodik (13) durch. Natriumfluorid zur Hemmung der N-AS-Acetat-Esterase wurde in der Menge von 1,5 mg/ml Inkubationslösung zugesetzt (8).

Naphthol-AS-D-Chloracetat-Esterase. Der Nachweis dieses Fermentes erfolgte nach Angaben von MOLONEY *et al.* (14).

Saure Phosphatase. Der Nachweis erfolgte gering modifiziert nach GOLDBERG und BARKA, wie von LEDER (11) angegeben.

Alkalische Phosphatase. Der Nachweis erfolgte in der von KAPLOW *et al.* angegebenen Methode (16).

Zum Nachweis der enzymatischen *Spaltung aminosäureartiger Bindungen* wurde die von SCHLAGER und KATZENSTEIN angegebene Methodik (19) gering modifiziert. Als Substrat verwendeten wir Alanyl-beta-Naphthylamid anstelle des von SCHLAGER und KATZENSTEIN angegebenen Leucyl-beta-Naphthylamids. Die Inkubationslösung wurde folgendermaßen hergestellt:

0,1 M Phosphatpuffer pH 6,5	30 ml
Propylenglykol	5 ml
D,L-Alanyl-β-naphthylamid HCl in Aqua bidest. gelöst (4 mg/ml)	15 ml
Echtgranatsalz GBC	120 mg
0,85 NaCl	30 ml
KCN 2 · 10 M	2 ml

Inkubiert wurde 4 h lang bei Zimmertemperatur.

Die *Sudanschwarz-Färbung* erfolgte mit Sudanschwarz B in einer gering modifizierten Methode nach MCMANUS (16).

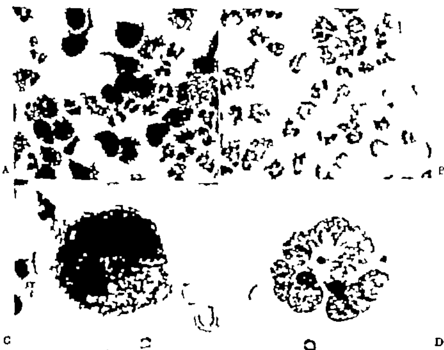


Abb. 1 Große Mononukleäre 10 h (A und B, Vergrößerung 400fach) und Riesenzellen (C und D Vergrößerung 1000fach) 76 h nach Anlegung des Hautfensters unspezifische Esterase-Reaktion ohne Zusatz von NaF (A und C) und Hemmung der Fermentreaktion in den Mononukleären und Riesenzellen durch NaF 1,5 mg/ml (B und D) (Naphthol-AS-Acetat, Echtblausalz BB e.k., Inkubation bei pH 6,8–7,0 für 70 min, Zimmertemperatur.)

Ergebnisse

Naphthol AS-Esterase In den in den ersten Stunden ausgewanderten Neutrophilen und vereinzelten Monozyten und Lymphozyten ließen sich dieselben Fermentaktivitäten wie in den entsprechenden Zellen des peripheren Blutes nachweisen In Hautfensterpräparaten, die mehr als 10–12 Stunden nach Beginn des Versuches abgenommen wurden konnte – selbst unter Berücksichtigung der stärkeren Abrundung vieler Zellen – eine deutliche Zunahme der nachweisbaren Naphthol-AS-Azetat Esterase in den großen mononukleären Zellen gefunden werden (Abb 1 A) In den Riesenzellen, die zu einem späteren Zeitpunkt auftreten, war ebenfalls eine starke Aktivität des Fermentes festzustellen (Abb 1 C) Durch Zusatz von Natriumfluorid zur Inkubationslösung (1,5 mg per ml) wurde eine Hemmung der Naphthol-AS-Acetat Esterase in

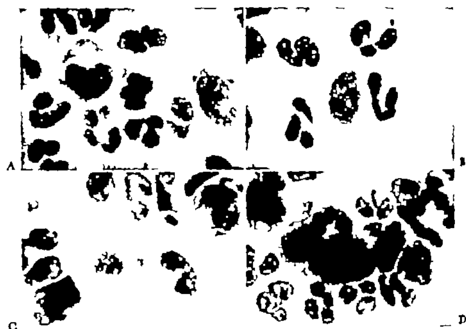


Abb. 2. Saure Phosphatase an Hautfensterpräparaten. A Monozyt 2 h nach Anlegung des Hautfensters (Vergrößerung 1000fach) B und C Mononukleäre nach 3 (B, Vergrößerung 1000fach) und nach 9 h (C, Vergrößerung 1200fach) D großer Mononukleärer 30 h nach Anlegung des Hautfensters (Vergrößerung 1000fach) (Naphthol-AS-Bi-Phosphat, hexazotiertes Pararosanilin, Inkubation bei pH 5,0 für 3 h bei 37 C)

Monozyten großen Hautfenstermononukleären und in den Riesenzellen bewirkt (Abb 1 B D) Die Esteraseaktivität in den Segmentkernigen und den vereinzelten Lymphozyten wurde durch Natriumfluorid nicht beeinflußt

Naphthol AS-Chloroazetat Esterase Die Fermentaktivität verhält sich bekanntlich völlig unterschiedlich von den unspezifischen Esterasen die mit Naphthol-AS-Azetat und alpha Naphthylazetat als Substrat nachweisbar sind Die segmentkernigen Neutrophilen wiesen eine starke die Monozyten eine sehr schwache und die Lymphozyten keine Anfärbbarkeit auf In den größeren mononukleären Zellen traten nach wenigen Stunden des Hautfensterversuches in Kernnähe deutlich nachweisbare Fermentaktivitäten auf die sich von phagozytierten Naphthol AS-D-Chloroazetat Esterase-aktiven Neutrophilenpartikeln unterscheiden ließen (Abb. 3 B und 4 B) Neben starker positiven Mononukleären fanden sich nach ca. 20 h immer noch Zellen die nur eine geringe bis fast negative Fermentreaktion aufwiesen



Abb 3. Hiläre Lokalisation von Fermentaktivitäten und Lipiden. A Mononukleäre Zellen 8 h nach Anlegung des Hautfensters, Peptidase-Reaktion (D,L-Alanyl-beta-naphthylamid, Echtgranatsalz GBC, Inkubation bei pH 6,5 für 4 h bei Zimmertemperatur Vergrößerung 1000fach) B Mononukleäre nach 5 h, Naphthol-AS-D-Chloracetat als Substrat (Echtgranatsalz GBC, Inkubation bei pH 7 4 für 30 min bei Zimmertemperatur Vergrößerung 1000fach), C Mononukleäre Zelle nach 10 h, Lipidnachweis mit Sudanschwarz B (Vergrößerung 1000fach)

Saure Phosphatase (Abb 2 Abb 4A) Bei den ausgewanderten Neutrophilen und Monozyten hat man zunächst den Eindruck daß die zytochemisch nachweisbare Fermentaktivität in den ersten Stunden in beiden Zellformen gegenüber den entsprechenden Zellen des Blutes etwas abnimmt. Später d h. in Präparaten, die ca 3 bis 10 h nach Anlegung des Hautfensters abgenommen wurden, fanden sich reichlich mononukleäre Zellen, die eine verstärkte Aktivität der sauren Phosphatase in Kernnähe, häufig im Bereich eines eventuell vorhandenen Kernhilus, aufwiesen. Allmählich nahm die Fermentaktivität weiter zu bis das ganze Zytoplasma, mehr oder weniger intensiv eine Aktivität des Fermentes erkennen ließ Ein ähnlicher Ablauf wurde von BRAUNSTEINER und DIENSTL (3) an peritonealen Makrophagen des Meerschweinchens beschrieben.

Peptidasen. Wir bezeichnen diejenigen Enzyme welche die säureamidartige Bindung von Naphthylamiden wie Alanyl-Leucyl oder Propyl-beta Naphthylamid und ähnliche spalten, als Peptidasen Es handelt sich hierbei nicht um spezifische Dipeptidasen, wie zum Beispiel die Leucinaminopeptidase. Die verwendeten Substrate werden von einer Vielzahl von Peptidasen und Proteinasen mit größerer oder geringerer Substrataffinität gespalten



Abb. 4 Phagozytose in den mononukleären Zellen des Hautfensters. A Saure Phosphatase, grobschollig verteilte Enzymaktivität in einer mononukleären Zelle. Präparat 9 h nach Anlegung des Hautfensters (Vergrößerung 1000fach). B Naphthol-AS-D-Chloracetat Esterase in ähnlicher Verteilung wie bei A, 10 h nach Anlegung des Hautfensters (Vergrößerung 1000fach)

Als Substrat verwendeten wir das Alanyl-beta Naphthylamid. Die Inkubationslösung wurde in Anlehnung an die von Schubert und Katzenmeier (19) angegebene Methodik hergestellt. Die Segmentkernigen zeigen mit diesem Substrat eine deutliche Fermentaktivität in den Blutausstrichen wie auch am Hautfenster. Monozyten waren ebenfalls deutlich positiv, während Lymphozyten nur eine ganz geringe Fermentaktivität erkennen ließen. Bei diesem Fermentnachweis hatten wir gleichfalls den Eindruck, als ob die Anfärbbarkeit in den ersten Stunden des Hautfensters in Segmentkernigen und Monozyten herabgesetzt wäre. Ähnlich wie bei der sauren Phosphatase kam es in den nächsten Stunden zunächst lokalisiert zu einer Zunahme der nachweisbaren Fermentaktivität. Schließlich trat in den großen Mononukleären eine sehr starke Zunahme ein (Abb. 3A). Riesenzellen wiesen ebenfalls eine deutliche Peptidaseaktivität auf.

Während die *alkalische Phosphatase* in den Segmentkernigen eine starke Aktivität erkennen ließ, konnten wir keine alkalische Phosphatase in den Mononukleären nachweisen (2, 11, 23).

Sudanschwarz B. Segmentkernige, Monozyten und Lymphozyten ließen am Versuchsbeginn dieselbe Anfärbbarkeit wie im

peripheren Blut erkennen. Drei bis 10 h nach Anlegung des Hautfensters fanden sich vermehrt mononukleäre Zellen mit einem deutlichen Gehalt an Sudanschwarz positiven Substanzen im Bereiche des Kernhilus (Abb 3C) In geringerer Anzahl fanden sich diese Zellen, neben diffus stark positiven auch zu einem späteren Zeitpunkt.

Diskussion

Zur Herkunft der Mononukleären Die Frage der Herkunft der Mononukleären konnte zunächst auch durch zytochemische Untersuchungen nicht geklärt werden BRAUNSTEINER *et al* (4 5) wiesen an der Ratte durch H^3 Thymidin Markierung der Zellen des strömenden Blutes nach, daß sich die im Hautfenster auftretenden mononukleären Zellen von Blutzellen ableiten. Durch diesen Nachweis war die Möglichkeit, daß Bindegewebszellen aus der Subcutis auswandern und sich zu den großen Mononukleären entwickeln, ausgeschlossen. LEDER *et al* (10 12) stellten in zytologischen und und zytochemischen (11) Untersuchungen fest, daß die Mononukleären in ihrer Fermentausstattung weitgehend mit den Monozyten übereinstimmten Auch WULFF gelangte zu ähnlichen Ergebnissen (23)

Nachdem FISCHER und SCHWALZL (8) nachgewiesen hatten daß die Naphthol-AS-Azetat Esterase Aktivität der Monozyten durch Natriumfluorid hemmbar ist, während Retikulumzellen verschiedenster Lokalisation, Bindegewebszellen, Neutrophile und Lymphozyten im Gegensatz dazu eine durch Natriumfluorid nicht hemmbare Esteraseaktivität enthalten, war es naheliegend, dieses Kriterium auch für die Mononukleären auf den Deckplättchen anzuwenden. Wie aus den Ergebnissen hervorgeht, ergab sich eine eindeutige Hemmbarkeit der Naphthol-AS-Azetat Esterase der Mononukleären des Hautfensters und der dort vorkommenden Riesenzellen Diese Zellen zeigten somit in dieser Hinsicht dasselbe Verhalten wie Blutmonozyten, während die Neutrophilen und Lymphozyten im Hautfenster ebenso wie im Ausstrich, keine Hemmung ihrer geringen Esteraseaktivität zeigten. Die von UNDRITZ (20) vertretene Hypothese der monozytären Herkunft der Mononukleären im Hautfenster scheint somit durch diese Ergebnisse bestätigt. Eigenartig ist allerdings die ausgeprägte Gestaltver

änderung insbesondere des Kernes welche diese Zellen nach ihrem Austritt aus dem Gefäß erfahren sowie die Tatsache, daß diese Zellen die Allergie vom verzögerten Typ in gleicher Weise wie Lymphozyten übertragen (5)

Funktionszustand der großen Mononukleären des Hautfensters. Eng verbunden mit dem Problem der Herkunft der Mononukleären des Hautfensters ist die Frage nach der Funktion der in den Zellen nachweisbaren starken hydrolytischen Aktivitäten. Eine Zunahme der in den Mononukleären nachweisbaren hydrolytischen Fermente fanden BRAUNSTEINER (2) sowie FISCHER *et al* (9) für die saure Phosphatase und spezifische Esterase und WULFF (23) für die Naphthol AS-D-Chloroazetat Esterase. Selbst unter Berücksichtigung der Tatsache daß häufig einzelne Zellen durch eine stärkere Abrundung eine größere Fermentaktivität vortäuschen konnte in der vorliegenden Untersuchung erneut eine Zunahme verschiedener hydrolytischer Fermente nachgewiesen werden Wie bereits ausgeführt, scheint diese Zunahme der Fermentaktivität bei einigen Enzymen an eine bestimmte intrazelluläre Lokalisation gebunden zu sein Saure Phosphatase und Peptidasen zeigten eine verstärkte Aktivität in einem umschriebenen, kernnahen häufig im Bereich eines Kernhilus gelegenen Zytoplasmabereich. Erst allmählich nahm im weiteren Verlauf die Fermentaktivität weiter zu, bis schließlich das ganze Zytoplasma vom Reaktionsprodukt ausgefüllt war Unter Berücksichtigung des Funktionszustandes der Mononukleären die eine sehr rege Phagozytose von Granulozytenteilen, Lipiden und auch von Tusche (siehe auch bei 9) aufweisen, er scheint es naheliegend diese Zunahme hydrolytischer Aktivitäten mit einem pH Optimum im leicht sauren Bereich auf eine Aktivierung der in den Lysosomen lokalisierten Fermentsysteme zurückzuführen. Es ist bekannt, daß in den Lysosomen einerseits saure Phosphatase andererseits solche Fermente vorkommen welche säureamidartige Bindungen zu hydrolysieren vermögen Der Lokalisation in Kernnähe und insbesondere in einem eventuell vorhandenen Kernhilus liegt die Beziehung zum Golgi-System nahe Nach neueren Arbeiten sollen die Fermente der verschiedenen Lysosomenformen in dieser Region ihren Ausgang nehmen (6) Für die unspezifische Esterase konnte in den Mononukleären keine derartige Lokalisation nachgewiesen werden

In Präparaten die zu einem sehr späten Zeitpunkt, z. B. 20 30 h nach Anlegung des Hautfensters, abgenommen wurden, kamen

neben den großen, sehr fermentreichen Mononukleären immer wieder mononukleäre Zellen zur Beobachtung, die nur eine geringe hilär lokalisierte Fermentaktivität erkennen ließen, beziehungsweise solche, die eine Enzymverteilung aufwiesen, wie sie frisch ausgewanderten Blutmonozyten entspricht. Es ist anzunehmen, daß nach dem primären Schub von Monozyten, die sich 4 bis 12 h nach Anlegung des Hautfensters zu den typischen Mononukleären entwickeln, auch in der Folge immer wieder Monozyten auswandern, die dann ebenfalls eine Transformierung erfahren. Nicht völlig geklärt ist die Abnahme der zytochemisch nachweisbaren Aktivität verschiedener Hydrolasen unmittelbar nach dem Auswandern ins Gewebe bzw. auf das Deckglas. Möglicherweise ist darin ein ähnlicher Vorgang zu sehen, wie er bei der Degranulation phagozytierender Neutrophiler morphologisch faßbar wird.

Die Naphthol-AS-D-Chlorazetat Esterase zeigte eine ähnliche Lokalisation wie die saure Phosphatase und die Peptidasen. Dieser Befund ist insofern auffällig, als dieses Ferment sein pH-Optimum nicht im sauren Bereich, sondern um den Neutralpunkt hat und sich dadurch von den lysosomalen Fermenten unterscheidet. Über die submikroskopische Zugehörigkeit der Naphthol-AS-D-Chloroazetat Esterase ist in der uns zugänglichen Literatur bisher nichts bekannt. Aus verschiedenen Beobachtungen neigen wir der Ansicht zu, daß dieses Ferment für die Phagozytosetätigkeit der Neutrophilen von Bedeutung ist.

Die Zunahme sudanschwarz-positiver Substanzen läßt sich auf Grund unserer Beobachtungen mit der Aktivierung der genannten Organellen gut in Einklang bringen. Cohn *et al.* (7) berichteten über Lipidtröpfchen in engem Zusammenhang mit dem hypertrophierten endoplasmatischen Retikulum in Kulturmakrophagen. Die Phagozytose steigert den Lipidgehalt dieser Zellen beträchtlich.

Zusammenfassung

An Hautfensterpräparaten wurden verschiedene zytochemische Fermentnachweise zur Darstellung der Aktivität von unspezifischer Esterase, Chloracylesterase, saurer Phosphatase, Peptidase und alkalischer Phosphatase sowie der Lipidnachweis mit Sudanschwarz B durchgeführt. Die starke Aktivität der großen Mononukleären und Riesenzellen beim Nachweis der Naphthol-AS-Acetat-Esterase läßt sich durch Zusatz von 1,5 mg/ml Natriumfluorid zur Inkubationslösung in beiden Zellformen fast völlig hemmen. Es besteht demnach eine Übereinstimmung dieser Zellen mit den Monozyten des Blutes. Die starke Zunahme hydrolytischer Fermente mit einem leicht sauren pH-Optimum und deren primäre Lokalisation im Bereich der Golgizone weist auf eine Steigerung der lysosomalen Ausstattung der großen Mononukleären hin.

Summary

Various cytochemical tests of the activity of unspecific esterase, chlor-acylesterase, acid phosphatase, peptidase and alkaline phosphatase and fat staining with Sudan black B were carried out on skin-window preparations. The marked activity of the large mononucleocytes and giant cells in the naphthol AS-acetate esterase test can be almost completely blocked in both cases by the addition of 1.5 mg/ml sodium fluoride to the incubation solution. These cells are then comparable to the monocytes in the blood. The considerable increase in hydrolytic enzymes at a slightly acid pH, and their primary location in the Golgi zone indicates an increase in lysosome in the mononucleocytes.

Résumé

Sur des frottis de cellules obtenues par la méthode de la fenêtre cutanée de Rebuck, différents examens cytochimiques ont été pratiqués afin de mettre en évidence l'activité de l'estérase non-spécifique, de la chloracyl-estérase, de la phosphatase acide et alcaline, de la peptidase, ainsi que le contenu en lipides par la coloration au noir de Soudan B.

La forte activité de la naphthol-AS-acétate-estérase des grandes cellules mononucléaires et des cellules géantes peut être presque complètement supprimée dans les deux types de cellules par l'adjonction de 1,5 mg/ml de fluoride de sodium à la solution d'incubation. Il existe donc une concordance entre ces cellules et les monocytes du sang périphérique. La forte augmentation des ferments hydrolytiques ayant un optimum de pH légèrement acide et une localisation primaire dans la zone de Golgi indique un accroissement de l'appareil lysosomal des grandes cellules mononucléaires.

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The Effect of Testosterone, Adrenal Steroids and Prolactin on Erythropoiesis

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Testosterone and adrenal steroids have been used to stimulate hemopoiesis in a variety of hematological conditions (1 2 3 4 5), and similar attempts to stimulate hemopoiesis in patients with bone-marrow failure have recently been made with prolactin (6) Testosterone (7–14) and prolactin preparations have also been shown to stimulate erythropoiesis in mice (7 8, 15 16) and rabbits (17)

In view of the effect of these hormones on erythropoiesis, the present study was undertaken to compare the hematological effects of *7-a methyltestosterone, ovine prolactin and prednisolone acetate* injected into female mice, alone, and in various combinations, over 15 days. In order to determine if these hormones could potentiate each other or the effect of erythropoietin, their erythropoietic effect, when given alone or in combination, was also studied in hypoxia induced polycythemic mice.

Materials and Methods

White CF 1 female mice weighing 20–25 g were divided into groups composed of minimum of 5 mice and were injected, daily for 15 days, with 250 µg of 7-a-methyl-testosterone propionate[1] 250 µg of ovine prolactin (NIH) 125 µg prednisolone acetate[2] or various combinations of these hormones, in the same doses. On the day following the

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7-a-methyltestosterone (Armour K88209) was kindly made available by Dr JAMES HOLLAND, Rochester N Y

Ovine prolactin, National Pituitary Agency NIH No. 1702A, was kindly made available by Dr E. E. McGARRY and Dr J C. BECK (300 I U /50 mg)

Prednisolone acetate (Schering No. 4 ACB 7)

last injection, the total blood volume (TBV) was determined by the I^{131}-tagged albumin dilution technique (RISA), from which the red cell volume (RCV) was calculated as previously described (15)

These hormones were also tested for erythropoietic activity in polycythemic mice by injection of test materials on the 4th and 5th days following removal from the hypoxic environment (posthypoxia) and the incorporation of Fe^{59} into erythrocytes determined at 72 h as described previously (8). All mice with hematocrits less than 50% were discarded.

The effect of the combined action of erythropoietically active agents was determined in polycythemic mice stimulated to secrete endogenous erythropoietin by re-exposing them to 10 per cent O_2 environment on the 4th posthypoxic day and injecting them with hormones as listed in Table I. Of those groups injected subcutaneously prior to exposure to 16 hours of hypoxia, those receiving 1 mg of 7-a-methyltestosterone in 0.1 ml sesame oil were injected 8 h prior to exposure, and those receiving 1 mg of ovine prolactin (NIH) in 0.1 ml saline were injected just prior to exposure.

All groups injected after exposure to 16 h of hypoxia received their injections immediately upon removal from the hypoxic environment. A control group of mice was injected with saline and exposed to 16 hours of hypoxia. The remaining groups were treated as shown in T ble I, being exposed to 8 h of hypoxia and/or injected with hormones on the 4th and 5th posthypoxic days. All groups were injected intravenously with 0.5 µc $Fe^{59}Cl_3$ 60 h after the end of exposure to hypoxia or following the last injection of test material, and the incorporation of Fe^{59} into erythrocytes was determined 72 h later

Results

The mean RCV of the mice injected daily for 15 days with 7-a-methyltestosterone and of those injected with prolactin was increased over that of the normal by 23% and 16% respectively Mice injected with both prolactin and 7-a-methyltestosterone increased their RCV by 37% indicating an additive effect of the combination of hormones (Fig 1) The prednisolone acetate treated mice lost weight and no significant increase of RCV was found when the reresults were calculated for the original body weight. Prednisolone acetate did not enhance or inhibit the effect of 7-a methyltestosterone alone and injection of all three hormones produced no further increase of the RCV over that produced by injecting prolactin and 7 a methyltestosterone.

Although all three hormones increased the plasma volume (PV) when injected independently the plasma volume increase was not reflected in a decrease of the hematocrit in the groups injected with prolactin or 7-a methyltestosterone due to the concomitant increase of the RCV The PV of mice who received various combinations of these hormones was not increased above those receiving 7-a methyltestosterone or prolactin (Fig 1)

Table I

The effect of injection of hormones in addition to the stimulation of endogenous e-thropoietin by the exposure of polycythemic mice to hypoxia.

Treatment	Incorporation of Fe^{59} into R of polycythemic mice (72 h)
Saline	0.63 ± 0.06
16 h hypoxia	13.48 ± 0.87
1 mg prolactin	1 74 ± 0.06
1 mg testosterone	1.67 ± 0.13
Hypoxia plus	
1 mg testosterone (prior)	36.06 ± 0.46
1 mg testosterone (post)	25.04 ± 1 75
Hypoxia plus	
1 mg prolactin (prior)	22.80 ± 0.94
1 mg prolactin (post)	19.26 ± 1 40
500 μg prolactin × 2	2.14 ± 0.12
500 μg testosterone × 2	1 73 ± 0.16
8 h hypoxia on two consecutive day	*11 40 ± 1.50*
plus	
500 μg prolactin × 2	26.20 ± 2.60
500 μg testosterone × 2	24 70 ± 1 46

Minimum of 5 mice per group. Mean ± S. E.
Prolactin (NIH)
7-a-methyltestosterone.

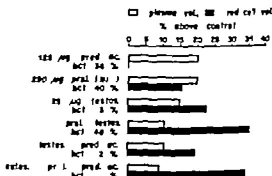


Fig 1 Per cent increase of plasma and red cell volume over that of the control followi injection for 3 weeks of prednisolone acetate 7-a-methyltestosterone propionate a pure prolactin alone and in combination.

The WBC counts as compared with the saline controls (mea 8.7/mm³) were unaffected by all combinations of hormones exce for those receiving 7-a methyltestosterone (mean 10.3/mm³) 7 a methyltestosterone and prolactin (mean 13 7/mm³)

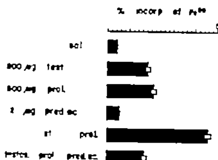


Fig. 2. Seventy-two-hour per cent incorporation of Fe^{59} into erythrocytes of polycythemic mice following injection of hormones.

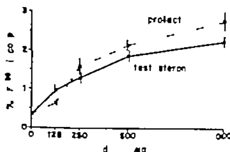


Fig. 3. Seventy-two-hour per cent incorporation of Fe^{59} into erythrocytes of polycythemic mice receiving prolactin (NIH) or 7-a-methyltestosterone. Vertical bar is the S. E. Each group was composed of minimum of 5 mice.

The dose response curves for prolactin and 7-a methyltestosterone are shown in Fig 3 The curves are similar and there are no significant differences between them at any dose level ($P > 0.05$)

Incorporation of Fe^{59} into erythrocytes of polycythemic mice following injection of 7 a methyltestosterone, ovine prolactin and prednisolone acetate alone, and in combination, is shown in Fig 2 The erythropoietic effect of prolactin and 7 a-methyltestosterone injected singly was similar and together they had an additive effect on the incorporation of Fe^{59} into erythrocytes. Injection of prednisolone acetate did not enhance the effect of 7 a-methyltestosterone alone.

The increased erythropoietic activity observed in polycythemic mice when these hormones were injected in conjunction with stimulation of endogenous erythropoietin by exposure to hypoxia, is shown in Table I One milligram of either 7-a methyltestosterone or prolactin, without exposure to hypoxia, produced a small ery

thropoietic response. When 7-a methyltestosterone was injected prior to, following or simultaneously with exposure to hypoxia, the erythropoietic response was much greater than that produced by hypoxia alone and was synergistic.

Injection of 1 mg of prolactin in the absence of exposure to hypoxia produced a response which was slightly less than that following the injection of 500 μg on two consecutive days ($P < 0.05$). Prolactin injected prior to or simultaneously with exposure to hypoxia, evoked a synergistic response. The response was less, but still synergistic, when it was injected after exposure to hypoxia. The effect of simultaneous injection of prolactin and exposure to hypoxia was equivalent to that produced by the same amount of 7-a methyltestosterone and hypoxia under similar conditions. There was less synergism produced by prolactin and the endogenous erythropoietin when the prolactin was injected prior to or following exposure to hypoxia than when 7-a methyltestosterone was injected into hypoxia stimulated mice under similar conditions (Table I).

Discussion

A REISFELD (18) preparation[4] of ovine prolactin (7, 8, 15, 16), a placental lactogen preparation (19) and testosterone (7, 8, 11–14) have previously been shown to increase the red cell mass and plasma volume of rodents and the incorporation of Fe^{59} into erythrocytes of polycythemic mice.

Prolactin (NIH) and 7-a methyltestosterone in combination increased the RCM and the incorporation of Fe^{59} into erythrocytes additively but their effect was not enhanced by the addition of prednisolone acetate which by itself had no erythropoietic effect.

The mode of action of these hormones has recently been under intense investigation with the presentation of evidence indicating that testosterone and prolactin may act on the renal production of erythropoietin. There is some indication, however, that testosterone may also act at the stem-cell level (20, 21). The clinically observed erythropoietic effect of testosterone in patients with bone-marrow failure and high plasma erythropoietin titers (22) would also appear to be due to an end organ effect rather than on the site of erythropoietin production. When the injection of these hormones was timed so that their absorption was occurring during the period of high plasma

[4] Ovine prolactin, Merck, 500 I. U./20 mg.

erythropoietin concentration and during its active utilization by the bone marrow a synergistic response was obtained, suggesting that under these conditions, the effect was at the bone-marrow level. The action of these hormones must therefore be evaluated at different levels in relation to a multisite effect rather than a specific action on one site. Experimentally the results obtained are related to the particular hormone preparation and its diluent, the time of injection, and the dose level.

The induction of erythropoietin production by the kidney perhaps by the potentiation of enzyme systems which are responsible for the elaboration of this hormone, is somewhat substantiated by the fact that testosterone is renotropic (23) and that prolactin increases renal plasma flow and glomerular filtration rate (24) However polycythemic mice have no detectable erythropoietin and few if any late erythroid precursors, and injection of these hormones alone or in combination produced only a slight increase in the incorporation of radio-iron into their erythrocytes. This could not be potentiated by a further increase of the dose of either hormone. The synergism produced by both of these hormones when injected not only prior to but also simultaneously with endogenous erythropoietin secretion 2½–3 days prior to injection of radio-iron, suggests they require a threshold of endogenous erythropoietin for their action to become fully evident. If they require the presence of erythropoietin, then one of their major sites of action would appear to be at the bone-marrow level. Their slight effect in polycythemic mice may be due to the potentiation of as yet undetectable amounts of endogenous erythropoietin

The possibility that these hormones also affect plasma protein binding of erythropoietin or alteration of the preformed complexed protein resulting in more efficient activation or release of erythropoietin, remains to be explored.

Summary

Both prolactin and 7-α-methyltestosterone, alone and in combination, increased the red cell volume and plasma volume of mice following their prolonged injection. Both increased the incorporation of Fe^{59} into erythrocytes of polycythemic mice and potentiated the effect of erythropoietin. Prednisolone acetate had no erythropoietic effect of its own and did not enhance the erythropoietic effect of testosterone or the combination of testosterone and prolactin. It is suggested that these hormones may act at several levels potentiating both the elaboration of erythropoietin and its action on the stem cell, but requiring its presence for their action to become apparent.

Zusammenfassung

Prolaktin und 7-α-Methyltestosteron führen allein oder in Kombination nach wiederholten Injektionen bei Mäusen zu einem Anstieg von Erythrozyten- und Plasmavolumen. Beide Hormone steigern bei polyzythämischen Mäusen den Einbau von Fe^{59} in Erythrozyten und potenzieren den Effekt von Erythropoietin. Prednisolonacetat an sich hat keinen Einfluß auf die Erythropoese und steigert den erythropoetischen Effekt von Testosteron oder einer Kombination von Testosteron und Prolaktin nicht. Es wird vermutet, daß diese Hormone an verschiedenen Stellen angreifen, indem sie sowohl die Produktion von Erythropoietin als auch seine Wirkung auf die Stammzellen potenzieren.

Résumé

Après leur injection répétée, la prolactine et la 7-α-méthyltestostérone augmentent données séparément et en combinaison le volume érythrocytaire et plasmatique. Les deux augmentent l'incorporation de Fe^{59} dans les érythrocytes de souris polycythémiques et renforcent l effet de l'érythropoïétine L acétate de prédnisolone pas d'effet érythropoïétique par lui-même et n augmente pas l effet érythropoïétique de la testostérone ou de la combinaison de la testostérone avec de la prolactine. Il est suggéré que ces hormones agiraient à différents endroits augmentant d'une part l'élaboration de l'érythropoïétine et renforçant d' utre part son action sur les cellules souches, la présence de celle-ci étant cependant indispensable à leur action.

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Die Isolierung funktionsfähiger Blutlymphozyten*

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In letzter Zeit finden *in-vitro*-Funktionen lymphatischer Zellen und damit Methoden zur Präparation möglichst reiner und ungeschädigter Lymphozytensuspensionen zunehmendes Interesse. Die bisher angegebenen Methoden gründen sich auf Zentrifugation im Dichtegradienten (1), Gelatinesedimentierung (2), Abtrennung der Granulozyten und Monozyten durch Eisenpulverphagozytose (3) sowie durch deren Adhäsion an Nylongewebe (4), Polystren (5), Glasflaschen (6), Glaswolle (7, 8) oder Glasperlen (8). Im weiteren beschreiben wir eine Methode der Lymphozytentrennung, die auf der Adhäsion der Granulozyten und Monozyten an Glasperlen (8) basiert. Sie gibt bei relativ geringem Arbeits- und Materialaufwand eine gute und weitgehend reine Lymphozytentrennung.

Material und Methoden

Im Prinzip wird eine mit kleinen Glasperlen gefüllte Säule mit leukozytenreichem Plasma beschickt, zwecks Adhäsion der Granulozyten und Monozyten an die Glasperlen bei 37 °C inkubiert und dann mit Plasma durchgespült.

Benötigt werden: Glaszylinder von 15 mm (9 mm) innerem Durchmesser und 16 cm (16 cm) Füllungslänge (Abb. 1); Glasperlen von 0,50 mm Durchmesser; Glaswolle; Gummistopfen mit Spritzenansatz. Die Glaswaren sind nicht silikonisiert.

Man füllt den Zylinder zwischen 2 Glaswollelagen von zirka 1 cm Dicke mit Perlen und setzt den Gummistopfen auf. Trockensterilisation ist einer Dampfsterilisation vorzuziehen. Alle weiteren Manipulationen sind unter sterilen Kautelen vorzunehmen.

Etwa 50 ml venösen Blutes werden mit 4 Tropfen konsolfreier Heparinlösung abgenommen und je nach Senkungsgeschwindigkeit ½ bis 1 h in Schrägstellung zur Sedimentation stehengelassen. Bei langsamer Blutsenkung setzen wir eine 5 %ige Gelatinelösung (Plasmagel, Laboratoire Roger Bellon, 159 Avenue de Roule, Neuilly, Seine, France) 3 ml pro 10 ml Blut zu (3) und lassen die Erythrozyten im Wasserbad bei 37 °C durch 30 min sedimentieren. Nach unserer Erfahrung ist streng darauf zu

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* Diese Arbeit wurde mit Unterstützung des Fonds "Kampf dem Krebs" durchgeführt.

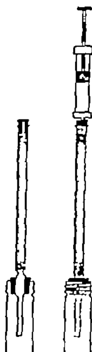


Abb. 1

achten, daß die Blutproben rasch verarbeitet werden, da sonst die Haftfähigkeit der Granulozyten abnimmt.

Der leukozytenreiche Überstand wird abgehoben und mit einer Spritze langsam in die sterile Säule eingebracht, die dann ½ h bei 37 °C inkubiert wird. Die von uns verwendeten perlengefüllten kleinen Säulen fassen etwa 6 ml, die großen ungefähr 10 ml Plasma. Durch scharfes Zentrifugieren des verbliebenen Blutes (10 min bei etwa 2.000 g) gewinnt man zellfreies Plasma, das zur Elution der Säule dient, aber auch durch heterologes Plasma ersetzt werden kann. Die Elutionsgeschwindigkeit soll zirka 20–30 Tropfen/min betragen. Übersteigt das Elutionsvolumen die ursprünglich eingebrachte Menge leukozytenreichen Plasmas (in unserem Falle 10 ml), so werden zunächst reichlich Blutplättchen und dann Granulozyten mitausgeschwemmt. Erythrozyten werden in der Perlensäule nicht retiniert und erscheinen daher immer mit den Lymphozyten im Eluat, lassen sich aber nötigenfalls unschwer durch Hämolyse in hypotonem Medium entfernen.

Kulturmethode

Die Zellen wurden nach der Isolierung unter sterilen Bedingungen in 80 × 26 mm Teströhrchen mit Schraubendeckel in Medium TC 199 und homologem Plasma (Endkonzentration 30%) bei Antibiotikazusatz (Penicillin 100 E/ml, Streptomycin 100 μg/ml) kultiviert (9). Die Zellzahl betrug zu Versuchsbeginn 10^6 Lymphozyten/ml Kulturansatz. Phytohämagglutinin (Wellcome) wurde in einer Endkonzentration von 0,01/ml zugegeben. 72 h nach Phytohämagglutininzusatz wurde der Prozentsatz transformierter Zellen im Ausstrich nach Beendigung der Kultur bestimmt.

In zahlreichen Versuchen hatten wir bei Kultivierung weißer Blutzellen von Normalpersonen ohne vorherige Lymphozytenseparation unter Phytohämagglutininstimulation nach 72 h eine Transformation von über 70% der Lymphozyten erreicht.

Ergebnisse

1 Reinheit der eluierten Lymphozytensuspension. Abgesehen von der Kontamination durch Erythrozyten und Thrombozyten, die sich nötigenfalls durch Hämolyse in hypotonem Medium bzw Differentialzentrifugation beseitigen läßt, erhielten wir eine sehr gute Lymphozytenanreicherung Durchschnittlich bestanden unsere eluierten Leukozytensuspensionen bei 20 Versuchen aus 98% Lymphozyten (98,0±0,9%) und zwar auch dann, wenn eine relative Lymphopenie vorlag (Tabelle I)

2 Lymphozytenausbeute. Von der Gesamtheit der in die Säule eingebrachten Lymphozyten ließen sich in 8 Versuchen nach Elution im Mittel 65% wiedergewinnen. Die Ausbeute war allerdings ziemlich variabel (65 6 ± 15,3%) Aus den großen Säulen erhielten wir pro Versuch zwischen 11 und 76 Millionen Lymphozyten.

Tabelle I

Vergleich zwischen einer Versuchsperson mit relativer Lymphopenie (Nr 321) und einem Probanden mit normaler Lymphozytenzahl (Nr 326)

Protokoll-Nr	321	326
Vor Separation		
Plasmamenge (ml)	10	10
Leukozyten (x 10^6/ml)	40,6	6,6
Gesamtzahl (x 10^6)	406,0	66,0
davon Lympho. (%)	5	33
Gesamtzahl Lympho. (10^6)	20,3	21,8
Nach Separation		
Plasmamenge (ml)	10	10
Leukozyten (x 10^6 ml)	1 15	1,50
Gesamtzahl (x 10^6)	11,5	15,0
davon Lympho. (%)	96	97
Gesamtzahl Lympho. (10^6)	11,0	14,6
Ausbeute (%)	54,2	67,0

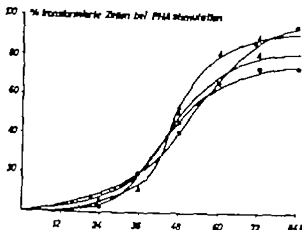


Abb. 2. Zeitlicher Verlauf der Phytohämagglutininstimulation ● ohne Granulozytenseparation, ▲ reine Lymphozytensuspension nach Säulentrennung

3. *Kultivierbarkeit.* Nach 72-stündiger Kultur unter PHA Stimulation fanden wir in 6 Versuchen im Mittel 81% (Bereich 67–90%) transformierter Lymphozyten. Auch im zeitlichen Verlauf der Transformation ergaben sich durch die Säulentrennung gegenüber der Kultivierung weißer Blutzellen ohne Lymphozytenseparation keine Unterschiede (Abb 2)

4 *Versuche mit anderen Methoden.* In Vorversuchen hatten wir die von COULSON angegebene Gelatinesenkung (2) die Abtrennung der Granulozyten durch Phagozytose von Carbonyleisenpulver (3) sowie durch Adhäsion an Breitwandkulturflaschen (6) versucht, dabei jedoch variable Ergebnisse erzielt und kaum einmal eine Reinheit der Lymphozytensuspension über 80% erreicht.

Diskussion

1 Nach Inkubation leukozytenreichen Plasmas in glasperlengefüllten Säulen kann unter bestimmten Voraussetzungen eine weitgehend reine Lymphozytensuspension eluiert werden (Anteil der Lymphozyten 98,4 ± 0 9% der eluierten weißen Zellen) Das Eluat enthält auch Erythrozyten und Thrombozyten, die eventuell durch weitere Präparation abgetrennt werden müssen. Die in der Säule zurückgehaltenen Granulozyten lassen sich durch Wechsel des Elutionsmittels nach RABINOWITZ (8) ebenfalls in weitgehender Reinheit gewinnen.

2. *Die Lymphozytenausbeute ist relativ gut und beträgt in* unseren Versuchen 65,6 ± 15,3% der in die Säule eingebrachten lymphatischen Zellen.

3. Die aus der Säule unter sterilen Kautelen eluierten Lymphozyten waren nach mehreren untersuchten Kriterien normal funktionsfähig. Ihre Stimulationsfähigkeit in der Phytohämagglutininkultur war nicht beeinträchtigt. Untersuchungen über RNS-Eiweiß- (10) und Lipidsynthese (in Vorbereitung), die an anderer Stelle mitgeteilt werden, waren weitere Hinweise auf die Lebensfähigkeit der nach der mitgeteilten Methode isolierten Lymphozyten.

4. Die Methode zeigt sich in unserer Hand verläßlicher als eine Reihe anderer zur Isolierung lymphatischer Zellen beschriebener Techniken.

Wir danken Frl. [illegible] *und Frl.* [illegible] *für ihre hervorragende* technische Mitarbeit.

Zusammenfassung

Es wird eine Methode angegeben, die mit relativ wenig Material- und Zeitaufwand eine Isolierung funktionsfähiger Lymphozyten aus Venenblut erlaubt und bei guter Zellausbeute ein weitgehend reines Lymphozytenkonzentrat liefert. Die Methode beruht auf dem Prinzip, leukozytenreiches Plasma in einer glasperlengefüllten Säule zu inkubieren, wobei die Granulozyten und Monozyten an den Perlen haften und bei der anschließenden Elution retiniert werden, sodaß im Eluat nur Lymphozyten, Erythrozyten und Thrombozyten enthalten sind. Rote Blutkörperchen und Blutplättchen können notigenfalls durch weitere Präparation entfernt werden.

Summary

A method is described for isolating viable lymphocytes from venous blood with little expenditure of time and material; an almost pure lymphocyte concentration is obtained with a high yield of cells. The method involves incubation of plasma containing large numbers of leukocytes in a column filled with glass beads; the granulocytes and monocytes adhere to the beads and are retained during the subsequent elution, so that the eluate contains only lymphocytes, erythrocytes and thrombocytes. The red cells and platelets can if necessary be removed by further preparation.

Résumé

Une méthode permettant l'isolation de lymphocytes viables à partir de sang veineux est décrite. Tout en ne demandant que peu de matériel et de temps, elle [illegible] un bon rendement et donne un concentrat très pure de lymphocytes. Elle consiste à incuber un plasma riche en leucocytes dans une colonne remplie de perles de verre, les granulocytes et les monocytes restant fixés aux perles de verre après l'élution consécutive de telle manière que l'éluat ne contient plus que des lymphocytes, des érythrocytes et des thrombocytes. Les érythrocytes et les thrombocytes peuvent, s'il est nécessaire, être éliminés par des procédés supplémentaires.

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A Simple Method for the Quantitation of Haemoglobin Fractions Obtained by Starch Gel Electrophoresis

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Zone electrophoresis on starch-gel is very useful for the separation of haemoglobin components. The qualitative results, obtained with borate or citrate buffers at alkaline pH. are really excellent however the quantitation of the individual fractions is very difficult. So far many different techniques have been proposed for this purpose GRATZER and BEAVEN's method (19) SMITH's two methods (33) the alpha amylase procedure, derived from PLAGEMAN GREGORY and WROBLEWSKI's method (27) for lacticdehydrogenase MORETTI BOURRIER and JAYLE's method (24 25) GOLDBERG and ROSS method (11 12) and SUNDERMAN jr's method (38 39) These techniques, which are the most important, are not wholly satisfactory except those of MORETTI *et al* and of SUNDERMAN jr SUNDERMAN jr's procedure is certainly valuable for A_2 quantitation in comparison with the remaining haemoglobin fractions considered together but it is not available for the other minor haemoglobin components (F_0, A_1) for the intermediate zones between the various fractions where some haemoglobin is present and for the so-called lectrophoretic tails. Moreover with this method the non haemic proteins which are occasionally present, cannot be detected. On the contrary the procedure of MORETTI *et al* is extremely exact Nevertheless we consider it too laborious for practical uses.

In this paper we shall attempt to describe a simple and practical procedure with which all the haemoglobin fractions, obtained by starch-gel electrophoresis, are dosed

Materials and Methods

Apparatus and hydrolyzed starch have been obtained by ELVI Soc., Milan I With this apparatus, four electrophoretic runs on the same gel can be realized.

Reagents

(A) Starch-gel buffer (17) 6 05 g of Tris and 0 78 g of disodium-EDTA are solved in about 2 l of distilled water; concentrate boric acid is added till pH 8 1 toward the volume is then adjusted to 2 l.

(B) Electrophoretic vessels buffer (17) 18.552 g of boric acid and 2.4 g of NaOH lets are dissolved in 1 l of distilled water. pH of this buffer is 9

(C) Staining solution 0.5 g of bromophenol-blue are dissolved in 100 ml of buffer (A).

(D) Decolorating and fixative mixture 100 ml of methanol are added to 20 glacial acetic acid and 80 ml of distilled water

(E) Benzidine-H_2O_2-reagent (17) 50 mg of benzidine are added to 70 ml of ethanol and 30 ml of acetate buffer 0.33 M, pH 4 7 0 2—0.3 ml 30% H_2O_2 are added to this solution when it is employed.

(F) 2.5 N NaOH solution.

(G) Glacial acetic acid.

For our researches red cells haemolysates from 30 healthy adults, from 25 umbilical cord blood of normal newborn babies and from 30 beta-thalassemia-trait carriers have been used. The haemolysates have been prepared immediately after the collection of blood samples, by lysing the washed red cells with distilled water. The remotion of stromal bodies was obtained with chloroform.

For haemoglobin electrophoresis on starch-gel, Smithies procedure (35, 36 37 modified by Boyer (7) was employed. Electrophoresis was carried out vertically using buffer (A) for the gel, and buffer (B) for electrophoretic chambers. This discontinuous buffer system is very advisable. The best results were obtained with runs of 16–20 h 200–300 V and 8–10 mA.

In order to dose all the haemoglobin fractions, present at the end of the electrophoretic run, some dyes, apt to be bound to the haemoglobin and proteins, have been tested. In particular amido-black 10 B, Ponceau S, light-green, luxamine, nigrosine and bromophenol-blue (6, 13 18) have been examined. It was found that all these compounds, except the last one, were unsuitable for our purpose. They are usually employed either in alcoholic, or acetic, or alcohol-acetic solutions. Therefore, they stain only the superficial layer, without diffusing into the gel. On the contrary bromophenol-blue, dissolved in distilled water (0.5% – w/v) diffuse completely into the gel, so that haemoglobin and protein fractions are entirely soaked. Since bromophenol-blue is also an indicator with colour change-zone between pH 3.2 and 4.6, it was dissolved in buffer (A). At this pH (8.1) bromophenol-blue solution was violet-blue and it preserved all its capacities to penetrate into the gel. All the haemoglobin and protein fractions were stained very well if the gel was immersed in the dye for an hour room temperature. The decoloration and fixation of the gel can be obtained by using the mixture (D). The gel must be left in it for 24 h at room temperature; the mixture must be removed and changed several times during this period. Bromophenol-blue stained not only the haemoglobin fractions, but also any protein component. To distinguish between non haemic and haemic components, the gel was cut horizontally into two slices of equal thickness. The lower slice was stained with bromophenol-blue and the upper slice with reagent (E). It was necessary to leave the upper gel slice in this reagent for several hours at room temperature. In this way it was possible to distinguish the haemoglobin from the non haemic fractions, because the latter were not stained with benzidine

T dose the fractions stain d with bromophenol-blue, the following procedure was used since four haemoglobin samples can be analyzed simultaneously with our pparatus, first of all, the strips of gel in which each haemoglobin sample had run, were divided by horizontal cuts thus, four strips of gel from the four different haemolysates were obtained. Next, all the strips were placed on glass with millimeter scale, and all the visible fractions of haemoglobin and proteins were removed from each strip by vertical cuts (Fig 1) In this way it was possible to measure the length and the position of each fraction on the scale, starting from the beginning of the run. Then, section exactly 10 mm long was excised from each strip in an area certainly free of dye, to be used as blank for spectrophotometric measurement. All the sections in which some dye was present, such as the intermediate zones between tw fractions and the so-called electrophoretic tails, were excised in the same manner Each fraction was eluted in 2.5 NaOH. A fraction 10 mm long was dissolved in 5 ml of hydroxide solution therefore, sections of different length were dissolved in amounts of hydroxide proportional to their respective lengths for example, fraction 5 mm long was dissolved in 2.5 ml of hydroxide 6 mm fraction in 3 ml, 7 mm fraction in 3.5 ml, and so on. The 10 mm long blank was obviously dissolved in 5 ml.

As soon as this step had been completed, the eluates were buffered with glacial acetic acid, by adding 1 ml of acid to each 5 ml of hydroxide. Therefore, the amount of acid added was proportional to the length of each considered fraction. As the alkali-acid reaction is exothermic, the test tubes must cool before spectrophotometric reading The bromophenol-blue-buffer (A) solution, at pH 8.1 showed the highest spectrophotometric bsorption at 588 nm wave-length. The stained eluates showed the highest spectral bsorption t the wave-lengths of 594 nm for HbA and 598 nm for HbF re spectively (Fig 2) Both followed exactly Beer Lambert law The spectrophotometric reading of each eluate and of the blank was carried out t the wa -length of 595 nm, against distilled water

For the calculation the procedure was the following If E = optical density of each eluate, B = optical density of the blank and mm = millimeters in length of each fraction, the planimetry of these is (E – B) × mm. If A = total planimetry of all the considered fractions, the percentage of each individual fraction is

$$\frac{(E-B) \times mm}{A} \times 100.$$

The percentage of electrophoretic components can be easily expressed on two carthesian axes in this case for each fraction, optical densities must be placed on the ordinate and length in millimeters on the bscissa. The optical density of the blank must of course be first subtracted from the optical density of all the fractions considered. The partial areas of each electrophoretic component are the product of the values concerning each optical density for the respective length (Fig 3 and 4)

Sources of Errors

(1) pH of buffer (A) pH 8.1 is the maximum that allows good separation of A_2 from NHP$_2$. With 8.05 pH, the separation is increased, but A_2 shows very little anodic mobility Therefore, to overtake these limits is not advisable

(2) Haemolysate dilution The solution of 10% Hb (±0.5) is the best one. I effect, with higher dilutions, t is difficult to dose the minor components on the other hand, with inferior dil tions, the spectrophotometric reading of the greatest fractions is not very correct. The same conditions take place if the amount of 10% haemolysate in the gel slits is either too little or too big. The most suitable haemolysate amount in each slit is 0.025 ml.

(3) Staining and decoloration of gel A good staining requires recent bromophenol-blue solution. Decoloration must be carried out as long as decolorating mixture is yellow

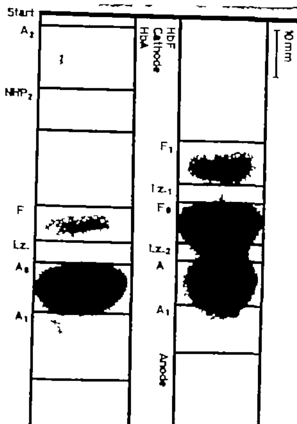


Fig. 1 Electrophoretic partition of haemoglobin. Right HbF Left HbA. Staining with bromophenol-blue. The straight lines indicate the cuts for excising each individual component. Migration is towards the anode.

(4) Gel cutting A greatest accuracy is necessary in cutting and in measuring each excised fraction. It would be advisable when it is possible, that the smaller fractions are as long as the blank (10 mm)

(5) Elution and spectrophotometric reading When each fraction is dissolved into the NaOH 2.5 N It is necessary to buffer it as soon as possible with glacial acetic acid. If buffering is not done immediately elution slowly decolorise

Reproducibility of the Results

In order to test it, we analysed the same HbA sample eight times on two different gels with four slits each Electrophoretic modalities and haemolysate amounts in all the slits were constant The results were the following

Fractions	NHP	A_2	NHP	F	Lz.	A_0	A_1	tot. A_0	tot. A
Mean	0.87	2.47	1.24	2.10	2.31	84.29	8.82	86.61	93.43
S. D. ±	0.084	0.157	0.085	0.069	0.151	0.262	0.282	0.252	0.168

Table I

Percentages of haemoglobin and protein electrophoretic fractions in normal adult and in umbilical cord blood

HbA 30 samples	I NHP	II A_3	III NHP	IV F	V z.	VI A_0	VII A_1	V+VI total A_0	V+VI+VII total A
Mean	bout 0.5–1	2.02	1.18	2.05	2.50	85.72	7.71	88.23	96.01
S. D. ±		0.68	0.36	0.57	1.29	2.97	2.77	2.63	0.94

HbF 25 samples	I F	II i. z_1	III F	IV	IVb i. z_2	V A_0	VI A_1	I+II+III+IV total F	IVb+V+VI total A
Mean	4.06	2.36	68.09	3.40	1.26	23.66	3.42	72.91	27.08
S D ±	2.32	1.09	4.36	1.58	0.72	3.42	1.24	3.75	3.64

Table II

Percentages of haemoglobin and protein electrophoretic fractions in beta-thalassemia-trait carriers

HbTh with increased A_2 10 samples	I NHP	II A_2	III NHP	IV F	V	VI A_0	VII A_1	V+VI total A_0	V+VI+VII total A
Mean	bout 0.5–1	4.80	1.99	2.28	2.03	82.48	8.46	84.51	92.94
S. D ±		0.48	0.49	0.68	0.89	2.47	2.29	2.53	[illegible]
HbTh with increased F 10 samples									
Mean	Id.	1.78	1.37	6.94	3.71	79.93	7.63	83.64	91.27
S. D ±		0.46	0.36	3.02	1.78	5.86	3.90	4.92	3.01
HbTh with increased A_2 and F 10 samples									
Mean	Id.	4.50	1.64	6.01	2.88	78.72	7.88	81.60	[illegible]
S D. ±		1.09	0.51	1.78	0.81	3.69	3.30	3.1	1.94

spectively in comparison with the remaining total fractions. From cathode to anode, haemoglobin spots and their respective percentages are the following A_2 = 2.02 %, F_0 = 2.05 %, A_0 = 85.72 %, A_1 = 7.71%. The amount of the intermediate zone between F_0 and A_0 (i. z.) is 2.50 but this component can be considered as a part of the greater A_0 fraction. Therefore, total A_0 is in effect 88.23 %. Total A is obtained by adding total A_0 and A_1: its mean value is 96.01 %.

Human foetal haemoglobin shows four electrophoretic spots; protein spots are absent. From cathode to anode, the position and

the percentage of each fraction are the following $F_1 = 4.86\%$ $F_2 = 68.09\%$ $A_2 = 23.66\%$ $A_1 = 3.42\%$. The intermediate zone between F_2 and A_2 (i. z_2) is divided into two unequal parts the first, which concerns F_2, is 3.40% the second, which concerns A_2, is 1.26%. Total F is 72.91% it can be calculated by adding F_1, i. z_1, F_2 and i. z_2 amount which concerns F_2. It seems correct to add all those foetal components, because they show the same alkali-denaturation rate. Furthermore, we observed that, with the electrophoretic partition of foetal haemoglobin-cyanide compounds, any F_1 fraction cannot be detected while F_2 values are proportionally increased.

Thalassemic haemoglobin shows four haemoglobin and two non haemic spots. In the first group of this haemoglobin type, A_2 percentage is 4.80%, whereas A_1 and total A values are proportionally reduced. In the second group F_2 percentage is 6.94% and A_2 and total A values are similar to those of the first group. In the third group, A_2 percentage is 4.50% and F_2 value is 6.01%. This group shows the smallest amount of A_2 and total A. The percentages of other haemoglobin fractions do not differ strongly from the normal values. NHP_2 is increased, particularly in the first and third group, where the greatest A_2 amounts are present.

Discussion and Conclusions

The proposed technique which has been utilized in our laboratory for over a year gives quantitative and analytical results very near to the data reported by many authors, who have used different procedures (1 2 3 4 5 11 12 16 17 20, 21 22 23 26 27 29 30, 31 32, 33 38, 39). This method completes the separative electrophoretic technique of haemoglobins on starch-gel. In fact, it makes possible to dose carefully each haemoglobin and protein fraction.

It is evident that this method is strictly subordinate to the separating possibilities of starch-gel electrophoresis, and that it is not free from sources of errors. Nevertheless, if it is applied exactly it allows the dosing with a sufficient rapidity and accuracy of all the fractions obtained on starch-gel electrophoresis and the results are highly reproducible.

For clinical purposes, this procedure gives valuable results, because the quantitative analysis of all the haemoglobin fractions allows the statistical elaboration of the obtained data.

Summary

A method for dosing haemoglobin and non haemic fractions obtained by starch-gel electrophoresis, at alkaline pH, is described. After showing the high reproducibility of the results, the obtained data, concerning HbA, HbF and HbTh, are related.

Zusammenfassung

Es wird eine Methode beschrieben zur Bestimmung von Hämoglobin und Nicht-Häm-Fraktionen, wie sie sich bei alkalischem pH mit der Stärkegel-Elektrophorese ergeben. Die Resultate sind gut reproduzierbar. Die erhaltenen Werte für HbA, HbF und HbTh werden angeführt.

Résumé

Une méthode servant au dosage de l'hémoglobine et des fractions non-hémiques telles qu'elles sont obtenues à un pH alcalin à l'aide de l'électrophorèse en gel d'amidon est décrite. Les résultats obtenus sont bien reproductibles. Les valeurs pour l'HbA, l'HbF et l'HbTh sont rapportées.

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Prevention of AKR Leukemia by Thymectomy at Varying Ages*

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The leukemia virus has been established in the AKR strain of mice as an etiological agent (1, 2). However, the nature of the role of the thymus in AKR leukemogenesis still remains to be clarified. In AKR leukemia, the thymus is almost invariably the primary site of the disease and the organ most severely involved (13, 14). Total thymectomy in young adulthood in this strain results in a marked decrease in the incidence of leukemias (5, 6, 16). Grafting of thymic tissue from AKR mice to thymectomized mice restores the susceptibility to leukemia development, and in many cases the tumors begin in the grafts themselves (3). Thymus grafts from histocompatible mice of low-leukemia strain have no such effect (4, 10). In the experiments with the F_1 hybrid mice between high- and low-leukemia strains, cytogenetic characters of leukemic cells in the lymphoma, developing in hybrids with thymus grafts, are in most cases, of the F_1 host, and not of the thymus donor (3, 15), suggesting that a leukemogenic influence of thymus grafts is non-cellular and indirect. However, since it has been demonstrated that in F_1 hybrids, lymphoid cells dividing in the parental thymus grafts become completely replaced by host type cells within 20 days after grafting (9), the genotype of leukemic cells does not give ample evidence for the existence of a humoral, indirect leukemogenic factor in the thymus. Metcalf (7, 8) has proposed the hypothesis that the thymus, probably the thymic medulla, is producing a humoral circulating, lymphocytosis stimulating factor, which exerts non-cellular influence on the lymphoid tissue responsible for leukemogenesis.

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Table II
Histological types of lymphomas developing in thymectomized and control AKR mice.

Group	No. of lymphomas	Lymphocytic type	Reticulum cell type*	Anaplastic type
3d–Tx*	0	0	0	0
35d–Tx*	3	0	2	1
150d–Tx*	4	0	2	2
180d–Tx*	5	3	1	1
Control	56	54	0	2

Thymectomy t 3 d, 35±5 d, 150±5 d, and 180±5 d of age.
See text (13 17)

One hepatoma, one hemangioma of the liver and one skin tumor were observed in thymectomized mice.

Histologically the authors divided lymphoma into three types lymphocytic, reticulum cell, and anaplastic type (13 14 17) The majority of lymphomas appearing in control AKR mice were of the lymphocytic type (lymphosarcoma and lymphocytic leukemia) It was reported previously that the histologic type, which is completely prevented by thymectomy is the lymphocytic type and that the other two types are less influenced (16) As shown in Table II out of 12 lymphomas developing in thymectomized mice, 5 were of the reticulum cell type with the average latent period of 389 d (298 to 428 d) and 4 anaplastic type with the average of 364 d (303 to 428 d) Three mice thymectomized at 180 d developed lymphocytic leukemia with the average latent period of 220 d (195 to 248 d) The greatest age at which thymectomy prevented completely the development of lymphocytic type was 150 d after birth. Since in the AKR strain a certain number of mice developed leukemia at about 180 d of age, it seemed that these three leukemias had already been generalized at the time of thymectomy

Discussion

It is clear from the above experiments that thymectomy performed at 3 35 150 and 180 d after birth is almost equally effective in preventing leukemia development in the AKR strain of mice. It *is doubtful, therefore, that the thymus is exerting systemic leukemogenic influence on the lymphoid tissue from the neonatal stage up* to the time of leukemia development. When such influence exists,

in the mice thymectomized at 150 or 180 d of age their thymic and extrathymic lymphoid tissues had been under the thymic, systemic leukemogenic influence until they were thymectomized and subsequently leukemia might develop from the extrathymic tissue in higher frequency in mice thymectomized at 150 or 180 d of age than in those thymectomized at 3 or 35 d. However this was not the case. An histological study in this laboratory on the earliest intrathymic leukemia gave evidence that leukemia usually first appeared less than one month prior to the clinical development of the disease (18) This age corresponds to 150–180 d after birth in younger individuals. Therefore it is apparent that thymectom at the preleukemic stage prevents leukemia development the sam as the operation in young adulthood Our recent studies suggest that the thymus provides a suitable environment for leukemia virus multiplication and that the virus multiplies in this organ to an amount which would be critical for the initiation of neoplastic transformation of thymic lmyphocytes at or after the age of about 5 months (18) It was of interest that lymphomas observed in thy mectomized mice were not of the lymphocytic type but of reti culum cell and anaplastic type. It seems likely from the present and previous experiments that the non-cellular humoral factor of the thymus, if it exists, is at least not a systemic, circulating factor but rather a local factor Whether or not a non-viral humoral leukemogenic factor exists in the thymus is not yet clear

Summary

Mice of the AKR strain were thymectomized at the following ages 3, 35, 150, and 180 d after birth. Thymectomy was, in all stages tested, effective in preventing leukemia development. The incidence did not differ significantly and was below 15 among all the groups of mice thymectomized at varying ages This result means that thymectomy at the preleukemic stage prevents leukemia development. The role of the thymus in connection with thymic humoral factor in leukemogenesis of AKR mice is briefly discussed

Zusammenfassung

Mäuse des AKR Stammes wurden im Alter von 3 35, 150 und 180 Tagen thymektomiert. I allen Fällen verhinderte die Thymektomie die Entstehung einer Leukämie Die Häufigkeit zeigte keine signifikanten Unterschiede und betrug bei allen Gruppen unter 15 Aus den Ergebnissen geht hervor daß eine Thymektomie im präleukämischen Stadium die Entwicklung einer Leukämie verhindert. Die Rolle des Thymus in Verbindung mit einem humoralen Faktor bei der Leukämieentstehung von AKR Mäusen wird kurz diskutiert.

Résumé

Des souris de la souche AKR furent soumises à l'âge de 3, 33, 1?0 et 180 jours à une thymectomie. Dans tous les cas, la thymectomie empêcha l'apparition d'une leucémie. L'incidence ne montra pas de différence significative et était en dessous de 13 tous les groupes. Il ressort de ces résultats que la thymectomie stade préleucémique prévient l' pparition d'une leucémie. Le rôle du thymus et d'un facteur humoral d'origine thymique dans la génèse de la souris AKR est discuté

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Sickle Cell Haemoglobin D Punjab Disease S from Ghana and D from England

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The first case of sickle cell haemoglobin D disease was reported by Itano in 1951 (1). Since that time 14 additional cases have been described (2–11). Although AD heterozygotes have been found in North Africa (12), West Africa (13) and the Congo (14), and there is a high frequency of S haemoglobin in West, Central and East Africa, only one case report of SD haemoglobinopathy has been published from Africa (9). The patient had S haemoglobin and haemoglobin D Ibadan, and did not show symptoms or signs of sickle cell disease.

In this paper, two cases with haemoglobin S and haemoglobin D Punjab are described. Both individuals showed a haemolytic anaemia and the elder was hospitalized for typical sickle cell crisis with hand-foot syndrome. The family was investigated and the haemoglobin D was fingerprinted. These cases raise some interesting questions concerning geographical distribution and chemical pathology.

Methods

The sickling test was performed by adding one drop of 2 sodium metabisulphite to one drop of blood and observing the preparation under a sealed coverslip. The foetal haemoglobin level was estimated according to the alkali denaturation technique of Singer *et al.* (15). Haemoglobin electrophoresis was carried out according to the method of Ringelhann and Marinoa using filter paper and agar gel at pH 8.6 and 6.1 (16, 17). The activity of G-6-PD was determined by the methaemoglobin reduction test of Brewer *et al.* (18) and the fingerprinting was carried out according to methods that have recently been summarized (9).

Case Report

J. H., tw year old girl was referred on the 9th of April, 1965 According to I parents, the child was sick with aches and pains fever and swelling of the fingers I toes. A sickling test was positive and electrophoresis on filter paper showed single ban I so that diagnosis of sickle cell anaemia was made.

On physical examination the infant was well developed, and although there pallor there was no icterus. The liver and spleen were not enlarged. Tachycardia present and the left foot was swollen.

Laboratory tests showed PCV 21 , WBC 75,200 including 326 nucleated red cells per 100 WBC. The differential count showed neutrophils 11 and lymphocytes plu nucleated red cells 89% No malaria parasites were seen. The blood of both parents and the propositus and her sister showed normal activity of G-6-PD On filter paper elec trophoresis both children showed single band and both parents double band

The child was put on promazine, 25 mg three times daily A eek later she re turned with fever and vomiting The urine showed trace of albumin, few pus-cells and bacteria. She was treated with sulphonamid (Gantrisin) but returned 10 da later with fever pains in the chest and abdomen. She was hospitalized and treated ith antibiotics (penicillin and tetracycline) After 5 days in the hospital she was allowed home On the 15th of July she had another ttack of fever and pains and the dose of promazine as raised to 150 mg daily until the 30th July when this was replaced by desmethylchlor promazine 50 mg daily By the 15th of November the dose of desmethylchlorpromazine had been reduced to 25 mg daily and subsequently this was discontinued.

The sister of the patient, A. H. who 11 months younger was investiga ed because of the illness of J H. There were no signs or symptoms of illness except for slight pallor There as no enlargement of the liver or spleen.

Laboratory Studies

On haematological examination the patient showed anaemia, moderate leukocytosis, reticulocytosis, aniso-poikilocytosis, and many oat shaped cells containing Howell-Jolly bodies. No target cells were seen. The younger sister showed mild anaemia and slightly increased reticulocyte count (T ble I). Both of the siblings and the mother had positive sickling tests but the father cells did not sickle. The foetal haemoglobin level was 5% in the patient and 20% in her asymptomatic sister The solubility of haemoglobin as highest in the father and lowest in the patient. The mother and the younger sibling had intermediate values for solubility of haemoglobin (T ble II)

On filter paper electrophoresis t pH 8.9 the patien had only single band in the S position. The younger sister had two bands one in the S position and the other be tween the S and A positions corresponding to haemoglobin F Both the father nd mother had tw bands in the A and the S positions Agar gel electrophoresis using alkaline buffer gav similar picture although the separa ion of S and F was clearer On agar gel electrophoresis using acid buffer both the patient and her sister showed two bands cor responding to the S and D positions (Fig. 1 and 2)

Fingerprints of tryptic digest of the Haemoglobin D were prepared and stained with ninhydrin and reagent specific for tyrosine. I was found tha the normal beta Tp XIII was been and new tyrosine positive peptide was present in the characteristic position of beta Tp XIII of Haemoglobin Punjab (Beta 121 Glu→Gln)

The laboratory results hich formed the basis for the diagnosis of sickle cell haemoglobin D disease are summarised in T ble III

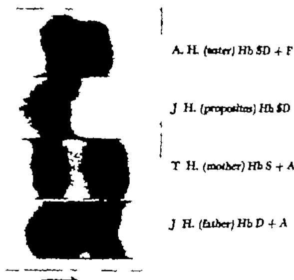


Fig. 1. Paper electrophoresis, pH 8.9 barbiturate buffer. The father's Hb D and the mother's Hb S move with the same speed. In the two children the D and S did not separate. In the blood of propositus A. H. there is also a distinct band corresponding to Hb F.

Family Studies

Mention has been made of the haemoglobin pattern of the propositus and her younger sister, the only children of a Ghanaian mother and an English father, whose haemoglobin patterns have also been described in the text. The father was born in England, his family having lived there for many generations. On his mother's side there was some Irish blood. The paternal grandfather showed an AA haemoglobin pattern. A paternal aunt showed AD haemoglobin pattern giving the impression that the paternal grand-mother had transmitted the abnormal haemoglobin.

Discussion

In sickle cell haemoglobin D disease there are some characteristic genetic, haematological and electrophoretic findings: (a) Both of the parents have one or more abnormal haemoglobins. (b) The blood of one of the parents and the blood of the patient give a positive sickling test. (c) The blood of the patient shows a single major band on paper electrophoresis at pH 8.6 and a double band on agar gel electrophoresis at pH 6.0–6.2. All of these criteria have been fulfilled in our cases.

Sickle cell haemoglobin D disease may be asymptomatic but is often characterized by articular pains, jaundice and weakness. Hepatomegaly, splenomegaly and leg ulcers may be present. Anaemia has been noted in all but two cases (9, 10). In some cases

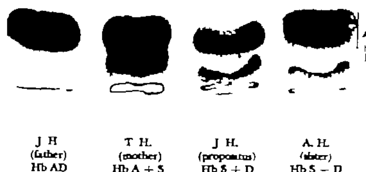


Fig 2. Agar gel electrophoresis, pH 6.2 citrate-citric acid buffer In the father blood A and D move with the same speed, in the mother blood A and S separate In the two children there is separate band for S and D In the blood of propositus A. H., the D band is very broad due to the juxtaposition of the F band.

there are typical sickle cell crises. The anaemia is usually microcytic and hypochromic although one case showed megaloblastic bone marrow (8) The blood film may show sickled cells and target cells.

The two cases described in this report did not have splenomegaly or hepatomegaly Only the older child had severe crises with fever anaemia and swelling of the extremities. The younger child was free of symptoms despite her anaemia. The most probable explanation for the difference in symptomatology is the difference in the level of foetal haemoglobin. JACKSON *et al* (19) found a favourable effect from increased levels of alkali resistant haemoglobin in sickle cell anaemia. CHARACHE *et al* (20) found that deoxygenation increased the viscosity of blood containing S haemoglobin but the F haemoglobin was almost incapable of interacting with S haemoglobin to increase the viscosity on deoxygenation. The more haemoglobin F is present in the red cell the less likely becomes sickling In sickle cell disease the distribution of F haemoglobin in the red cells is not uniform as in the case of high F gene

Most of the cases of sickle cell haemoglobin D disease ha e occurred in non Negro patients. Although haemoglobin D occurs in Africans it is also present in English, French, and Portuguese the highest incidenc being found in northern India, the Punjab where it is found in 3 of persons. Thus the D gene as in our cases, may be derived from Europe or India, although in one case reported from

Table I

Name	PCV %	WBC %	Retic.	Morphology
J. H. (father)	46	5.500	0.8	N. A. D.
T. H. (mother)	37	9.200	1.6	moderate hypochromia
J. H. (propositus)	25	11.900	10.0	aniso-poikylocytosis sickle shaped cells, Howell Jolly bodies, 98 N.RBC/100 WBC
A. H. (sister)	20	4.200	4.0	Polychromasia

Table II

Name	Hb. type	Foetal haemoglobin %	Haemoglobin solubility
J. H. (father)	AD	0.5	100
T. H. (mother	AS	0.5	41
J. H. (propositus)	SD	5.0	25
A. H. (sister)	SD	30.0	59

Table III

Name	Electrophoresis alkaline paper	acid agar	Sickling	Haemoglobin solubility	Foetal haemoglobin
J. H. (father)	two bands	one band	negative	high	low
T. H. (mother)	two bands	two bands	positive	low	low
J. H. (propositus)	one band	two bands	positive	very low	high
A. H. (sister)	one band	two bands (+F)	positive	low	very high

Africa it appears likely both the S and the D genes were of indigenous origin (9). In this case the D was D Ibadan and not D Punjab.

In the case reported by Smith and Conley (5) the type of D haemoglobin was determined by fingerprinting by Baglioni (21) and it was haemoglobin Punjab. In the case reported by Watson-Williams et al. haemoglobin D Ibadan was identified (9). The ... of Smith and Conley and our two which had D Punjab sh... anaemia while the case with haemoglobin D Ibadan did

Hb. D from Accra

αTpXXI

β^DTpXIII

β^ATpXIII missing

− +

Fig 3. Fingerprinting of Hb D. The normal beta Tp XIII is absent and new tyrosine positive peptide is present in the characteristic position of beta Tp XIII of Haemoglobin D Punjab

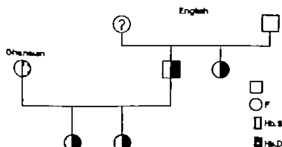


Fig 4. Family tree.

anaemia. RESTREPO and LONDONO reported a case that did not have anaemia, but fingerprints were not reported (10). It is possible that the type of haemoglobin D influences the symptomatology in sickle cell haemoglobin D disease. The study of such interactions may further our understanding of sickle cell disease.

Summary

Two female siblings with haemolytic anaemia are described, one aged 2 years, the other 11 months. The older child had symptoms of sickle cell crises with hand foot syndrome. Blood from both siblings showed the presence of haemoglobin S and haemoglobin D as well as 5% foetal haemoglobin in the older child and 20% in the asymptomatic sibling. The mother, a Ghanaian, had sickle cell trait and the father, an Englishman, haemoglobin D trait. The haemoglobin D was identified as haemoglobin D Punjab by fingerprinting. The interaction of haemoglobin S and various haemoglobins D is discussed.

Zusammenfassung

Es wird über zwei Schwestern im Alter von 2 Jahren bzw. 11 Monaten mit hämolytischer Anämie berichtet. Das ältere Kind zeigte Symptome einer Sichelzellkrise mit Hand-Fuss-Syndrom. Im Blut beider Geschwister wurden Hämoglobin S und D nachgewiesen, sowie beim älteren Kind 5% und beim jüngeren, asymptomatischen Kind 20% Hämoglobin F. Die Mutter, eine Ghanaerin, war Trägerin des Sichelzellmerkmals, der Vater, ein Engländer, war Träger des Hb D-Merkmals. Mit der Fingerprint-Methode konnte das Hb D als Hb D Punjab identifiziert werden. Die Wechselwirkung zwischen Hb S und verschiedenen D-Hämoglobinen wird besprochen.

Résumé

Les cas de deux sœurs atteintes d'anémie hémolytique dont l'une était âgée de deux ans et l'autre de 11 mois sont rapportés. La plus âgée des deux enfants présenta les symptômes d'une crise drépanocytaire avec un syndrome main-pied. Dans le sang des deux sœurs se trouvèrent de l'hémoglobine S et D ainsi que 5% d'hémoglobine foetale chez la plus âgée et 20% chez la plus jeune qui elle ne présentait pas de symptôme. La mère, une ghanaéenne, était porteuse du trait drépanocytaire et le père, un anglais, porteur du trait de l'hémoglobine D. L'hémoglobine D a été identifiée à l'aide de la méthode du «fingerprint» (dite de l'empreinte digitale). L'interaction de l'hémoglobine S avec différentes hémoglobines est discutée.

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An Example of Abberant Blood Group (B?)

P K. Raha, H K. Sarkar and C. L. Mukherjee

In adult human RBC expression of B antigen in weaker form is very rare as stated by Mollison (6) Recent categorisation of the subgroups of B by Alter and Rosenfield (1) have been per formed from the very few observations on weak blood group B

This case named Warrior is possibly the first where the available means failed to identify the blood group antigenic char acter

Materials and Methods

Blood sample of Warrior true hermaphrodite was sent for routine grouping, when categorisation of the blood sample to specific ABO group could not be done Necessarily this led to perform series of tests.

Red cells and serum were tested against 10 differant batches of anti A, anti B, anti A-B and known A cells, B cells respectively in the usual tube test procedure As the serum agglutinated all the A cells, not the B cells and there was no gglutination with anti A, anti B and anti A B, it was contemplated that this may be weaker form of blood group B. the followings tests were also performed.

With 3 batches of an B of known titre, 2 batches of B cells and test cells were incubated t room temperature for 30 min, centrifuged and the supernatants were diluted two folds Then the same groups of cells in 2% saline suspension ere added and kept at room temparature, with the idea to note the drop in the original titres of anti B (T ble I

Saliv and serum of Warrior was tested for B substance and H substance as outlined by Kab 4)

In the usual antiglobulin (Coombs) est procedure the test cells were tested against anti A, anti B and anti A B.

Elution stud ccording to Sussman et al (9) agglutination test with anti B and anti A-B using 4 gum accacia solution as outlined by Alter and Rosenfield (2), and with trypsinized cells as stated by Mollison (6) were also tried

Vander Hart et al 10 performed test to detect these weaker blood groups here the test cells and control group 0 cells were washed treated with an B. washed treated with A-B substance (Neutr AB Dade reagents Inc. Miami and this was repeated three times, lastly with anti B was left overnight 4 C

As the last resorts to watch whether or not, there is rapid destruction of these cells ben labelled cells are injected in group 0 olunteers as indicated by MOLLISON (7 and to watch the stimulation of formation of anti B perchance by injecting the est cells rabbits as pointed out by LEVINE (5) could not be performed

Rh and other blood group characteristics were performed with the help of re spective anti sera (supplied by Dade reagents Inc. Miami) They are summarised in Table II

Results

The red cells did not show any agglutination with the series of anti A, anti B and anti A B but the serum agglutinated only the A cells, not the B cells The cells were agglutinated with anti H serum obtained from human source reported by RAHA *et al* (8)

Saliva and serum was tested for B substance and H substance and they were found to be absent

Anti B and anti A B sera were incubated along with the patient s washed cells at 37 C for 2 h with saline and AB serum control Cells were then washed thrice and anti human globulin (Coombs) sera were added and centrifuged, there was no agglu tination in either test or control tubes

Elution study was performed with anti B and anti A B, patient s cells were incubated at room temparature for 2 h, then the cells were washed 4 times. After that the supernatant were discarded and equal volume of cold saline was added which was incubated at 56 C for 15 min. To the supernatant after being centrifuged, known 2 B cells and patient s cells were added in differant sets kept at room temperature for 1 h and then centrifuged There was no agglutination in either tubes.

Test red cells were incubated with known anti B for one hour at 37 C, the supernatant was discarded. After addition of 0 04 ml of 4 Gum Accacia solution, the tubes were centrifuged then kept at 37 C fo 15 min and lastly again centrifuged. This also did not bring about any agglutination.

After trypsinisation, cells were incubated with anti B and anti A B sera, not resulting into any agglutination

Control group 0 cells and test cells were washed, treated with anti B washed treated with AB substance and washed, this was repeated 3 times, lastly with anti B it was left overnight at 4 C. There was no agglutination in th test and control cells.

Other blood group characteristics are summarised in Table II

The genetical study is being done by another group of workers The chromosome pattern of this case was x/xxy

Table I
Titre of different batches of anti B when tested with the same batches of B cells for the second time

Anti B	Original titre	B cells (193)	B cell (195)	Test cell
1st batch	1/64	1/4	1/2	1/32
2nd batch	1/32	1/2	1/2	1/16
3rd batch	1/32	1/2	1/2	1/16

Table II
Other blood group characteristics.

Rho	Rh phenotype	M	N	S	s	P	Le^a	Le^b	Fy^a	K	k
+	CCDee	+	+	—	—	+	—	—	+	—	+

Table III
Serologic characteristics of the type of blood group B.

Hemagglutination				Hemagglutination Inhibition by Secretor Saliva	
Phenotypes	anti A	anti B	anti A, B	anti A	anti B
B1	N	S	S	N	S
B3	N	M	M	N	W
Bx	N	VW	W	N	N
Bg	N	VW to W	W	N	S
Bm	N	N	VW	N	S

N No agglutination or inhibition, *S* strong agglutination or inhibition,
M mixed or weak agglutination with unagglutinated cells,
W weak agglutination, *VW* very weak agglutination.

Discussion

Weak form of blood group B is very rare. The serologic characteristics of those rare subgroups of B can be quoted (Table III) from the report of ALTER and ROSENFIELD (1)

Though in this particular case the family study could not be performed as the patient belongs to a different state of India, came to Calcutta only for tratment of hermaphroditism but nevertheless the series of tests that were performed with the patients blood brought about very interesting observations. Anti A was present in

the serum, anti B was absent but the red cells of the patient did not give any agglutination neither with anti A anti B or anti A B The agglutination test performed at two stages (Table I) the indirect antiglobulin test with anti B and anti A B elution study test with 4 % gum accacia and trypsinised cells, test with latticed cells did not bring about any evidence of the presence of a very weak form of B group antigen in red cells. Injection of labelled test red cells in human volunteers and test red cells into rabbits could not be performed.

The classification was further complicated as saliva and the serum of the patient found out to be nonsecretor of H substance (as also B substance)

The cells were agglutinated by anti H serum. The negative tests with anti S and anti s serum possibly denotes that the patient belongs to $S^{u}S^{u}$ type The Le a-b fits in with the patients non secretor state, homozygous se se type.

The case reported by ARMSTRONG *et al* (3) also a true hermaphrodite where family study could not be performed, did not demonstrate any B antigen in red cells even by elution tests but B substance was present in saliva The case reported here is also similar to that but the situation of typing the ABO blood group was further complicated as the patient happened to be a non secretor

So in this particular case the ABO blood group of this case can be designated as group B?

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Summary

ABO blood group antigen of red cells of non secretor true hermaphrodit could not be ascertained while the serum agglutinated only A cells The blood group was designated as group B

Zusammenfassung

Bei einem -bten Hermaphroditen und Nichtsekretor konnte das ABO-Blutgruppenantigen der Erythrozyten nicht ermittelt werden, während das Serum nur A-Zellen agglutinierte Die Blutgruppe wurde als B bezeichnet.

Résumé

Chez un hermaphrodite authentique et non-sécréteur l'antigène de groupe ABO des érythrocytes ne put être mis en évidence. Le sérum agglutinant cependant uniquement les cellules A. Le groupe sanguin fut dénommé B

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Knochenmarkschäden nach beruflicher Einwirkung des Insektizids gamma Hexachlorcyclohexan (Lindan)

R. Stieglitz, H. Stobbe und W. Schüttmann

Beim Vorliegen von Panmyelophthisen und Panmyelopathien können in Einzelfällen physikalische (ionisierende Strahlen), toxische (z. B. Benzol) oder allergische Noxen ausfindig gemacht werden, denen eine kausale Bedeutung beizumessen ist. Die überwiegende Zahl der Fälle bleibt jedoch noch immer ätiologisch ungeklärt. Es muß die Vermutung ausgesprochen werden, daß sich in dieser Gruppe Fälle verbergen, bei denen ursächliche Umweltfaktoren nur deshalb nicht ermittelt werden, weil entweder nicht nach ihnen gesucht wird oder weil ihre pathogene Wirksamkeit nicht zum allgemeinen Wissensgut der Ärzte gehört.

Dies mag in besonderem Maße für das Insektizid gamma Hexachlorcyclohexan (HCH) gelten, das unter der internationalen Bezeichnung Lindan bekannt ist. Während im Ausland, besonders in den USA, eine Anzahl von hämatologischen Systemerkrankungen als Folge einer Lindaneinwirkung veröffentlicht wurden, fehlen im deutschsprachigen Schrifttum bis auf eine Erwähnung durch Behrbohm (2) entsprechende Veröffentlichungen, obwohl Moeschlin (19) in seiner Monographie bei Nennung der verschiedenen Gifte, die aplastische Anämien hervorrufen können, HCH anführt. Unsere Mitteilung soll die Aufmerksamkeit auf die Tatsache lenken, daß auch in Mitteleuropa derartige Krankheitsbilder zu beobachten sind.

Beim HCH handelt es sich um eine chlorierte Kohlenwasserstoffverbindung, deren insektizide Wirksamkeit in den ersten Jahren des letzten Krieges unmittelbar nach der des DDT (Dichlordiphenyltrichloräthan) von französischen und englischen Autoren wiederentdeckt wurde, nachdem 1933 eine entsprechende Mitteilung unberücksichtigt geblieben war. Nach Kriegsende fand das HCH neben dem DDT in der ganzen Welt als Bestandteil von Schädlingsbekämpfungspräparaten weite Verbreitung.

Bereits 1953 hatten DANOPOULOS *et al.* (5) bei den von ihnen beobachteten subakuten HCH Vergiftungsfällen auf Störungen der Blutbildung, besonders der Erythrozytopoese hingewiesen. Im Knochenmark ihrer Kranken glaubten sie eine Markhemmung festgestellt zu haben. Im gleichen Jahr wies das «Commitee on pesticides» der USA aufgrund mehrerer Beobachtungen daraufhin, daß das HCH im Knochenmark die Blutbildung, insbesondere die Erythrozytopoese zu hemmen vermag (3)

MUIRHEAD *et al.* (21) haben bei einem Landwirt mit Exposition gegenüber HCH-verwandten Insektiziden eine hämolytische Anämie mit positivem Coombstest nach Zugabe von Zubereitungen der entsprechenden Insektizide beobachtet. Als für kurze Zeit eine erneute Exposition gegen Pflanzenschutzmittel stattfand, kam es zu einem Rückfall. HARGRAVES (12) erwähnt unter 15 Beobachtungen von hämatologischen Systemerkrankungen, die er auf die *Einwirkung von Umgebungsnoxen zurückführt*, 4 HCH bedingte Fälle.

Häufiger und in ihrer Symptomatik eindrucksvoller sind Berichte über Panmyelophthisen und Agranulozytosen durch HCH Einwirkung (Tabelle I) Die angeführten Mitteilungen über hämatologische Erkrankungen nach HCH Einwirkung der Tabelle I sind nur Beispiele, die aus der Literatur ausgewählt wurden. Auch die *Halbjahrestabellen* der Studiengruppe für *Blutkrankheiten* des Rates für Arzneimittel der American Medical Association (4) in denen seit 1957 auch HCH-Schäden registriert werden, erfassen nach Meinung der Herausgeber naturgemäß nicht alle auftretenden Fälle, da sie auf den freiwilligen Berichten beruhen, die dem Rat für Arzneimittel von den Behandlungsstellen zugehen. Bevor sie in die Tabelle aufgenommen werden, erfolgt eine eingehende Prüfung Aus diesen Gründen sind die in der Halbjahrestabelle 1963 (Tabelle II) zusammengestellten Fallzahlen für HCH besonders beachtenswert.

Wir konnten innerhalb weniger Jahre insgesamt sechs Beobachtungen registrieren, bei denen die hämatologische Erkrankung mit HCH Einwirkung in Zusammenhang gebracht wurde.

Tabelle 1
Mitteilungen über Panmyelophthisen und Agranulozytosen durch HCH-Einwirkungen.

	Zahl der Fälle	Erkrankung	Exposition	Besonderheiten, mögliche andere Krankheitsursach… Verlauf
…und …ers (10)	1	Panmyelophthise	59jähriger Landwirt, der unmittelbar vor der Erkrankung zwei Tage bei ungünstigen Windverhältnissen ein 10% gamma-HCH enthaltendes Präparat verstreut hatte	Exitus letalis
…er und …7) 1955	1	Panmyelophthise	16jähriger Junge, der 2 Jahre lang vor Krankheitsausbruch bei der Behandlung von Viehherden Kontakt mit lindanhaltigen Insektiziden hatte	Exitus letalis
…20) 1955	1	Panmyelophthise	Zweijährige massiv Exposition gegen HCH, DDT und Chlordan	Exitus letalis
…27) 1955	1	Panmyelophthise	Starke HCH-Exposition ohne besondere Angaben	Exitus letalis
…an et al. …5	1	Agranulozytose	45jähriger Kleiderlagerverwalter der mehrere Monate kristallinen gamma-HCH-Nebeln ausgesetzt war	Heilung nach Kortisontherapie
…er et al.	1	Panmyelophthise	43jähriger Desinfektor der seit 3 Jahren mit Lindan gearbeitet hatte	erfolgreiche Therapie m… Steroiden
…8) 1959	1	Panmyelophthise	Arbeiter bei der HCH-Herstellung mit sehr intensiver Exposition	Exitus letalis
…ox (2)	3	Panmyelophthise	als Landarbeiter und Pflanzenschutzhelfer DDT und HCH Mischpräparaten ausgesetzt	Exitus letalis
		Panmyelopathie	als Pflanzenschutzwart DDT und HCH exponiert	Wiederauftreten der hämorrhagischen Diathese e… erneutem DDT/HCH Kontakt folgend
		Panmyelopathie	als Zimmermann mit DDT/HCH-haltigen Holzschutzmitteln gearbeitet	gleichzeitige Hepatopathie
…z et al. …63	20	Panmyelophthisen	innerhalb der letzten 6 Monate vor Auftreten der klinischen Krankheitserscheinungen hatten alle wiederholten Kontakt mit hauptsächlich HCH und DDT-enthaltenden Präparaten	lediglich in 4 Fällen beste… außerdem eine möglicher… weise bedeutsame ander… weitige Exposition
…15) 1963	2	Panmyelophthise	8jähriges Mädchen, das bis zum Krankheitsbeginn zwei Jahre hindurch etwa 2 h täglich in einem Raum gespielt hatte, in dem ganz tägig ein Lindanverdampfer tätig war	äußerst massive Exposition Exitus letalis
		Panmyelophthise	52jähriger Buchhalter der 3 Monate einen Lindanverdampfer auf dem Fernsehgerät in Betrieb hatte	äußerst massiv Exposition Exitus letalis

Tabelle II

Halbjahrestabelle 1963 der Blutschäden durch Chlordan, DDT und Lindan.

Mittel	Aplastische Anämie[1] mit Panzytopenie			
	Mittel	mit anderen Arzneimitteln		
	allein	harmlos*	unverdächtig*	toxisch
Chlordan	3	1	3	5
Dichlor-diphenyltrichloräthan (DDT)	3		5	11
Gamma-Hexachlorcyclohexan (Lindan)	7		3	3

Aspirin, Barbiturate, Chloralhydrat, Digitalis, Penicillin, Tetracyclin.
Nicht bekannt, daß sie Bluterkrankungen hervorrufen.

Kasuistik

Beobachtung 1 W. S., geb. 1892. Bei dem 66jährigen Gärtner traten Anfang August 1958 Nasenbluten und an den Unterschenkeln und Oberarmen große blaue Flecken auf. Kleinere Verletzungen bluteten auffallend lange. Wegen dieser Erscheinungen wurde er vom 8. August bis 17. September 1958 in einem Bezirkskrankenhaus behandelt. Bei der Aufnahme fanden sich flächenhafte sowie punktförmige Haut- und Schleimhautblutungen. Das Hämoglobin war auf 40% abgefallen. Die Thrombozytenzahl betrug 17000/µl. Im Sternalmark zeigte sich ein Fehlen der Megakaryozyten. Alle anderen Zellelemente waren annähernd normal vorhanden. Da der Pat. ausschließlich Kontakt mit dem HCH-haltigen Präparat Arbitex® angab, wurden Thrombozytenagglutinationsteste durchgeführt. Patientenserum agglutinierte Fremdthrombozyten bei Zugabe von Arbitex. Nach Inkubation bei 37°C war die Agglutination verstärkt. Sie war auch mit hitzeinaktiviertem und mit durch Bariumsulfat vorbehandeltem Serum des Patienten nachweisbar, so daß Gerinnungsfaktoren als Ursache einer als falsch positiv zu deutenden Gerinnungsagglutination auszuschließen waren. Parallele Versuchsansätze mit DDT (Wofatox®) und verschiedenen Düngemitteln fielen negativ aus. Diese Untersuchungsergebnisse bestätigten die allergische Natur der Thrombozytopenie. Behandlung mit ACTH und Bluttransfusionen führte zu einer raschen Heilung. Dem Patienten wurde eine weitere Beschäftigung mit Pestiziden untersagt. Nach einem flüchtigen erneuten Kontakt mit HCH bei der Übergabe des Pestizidlagers an den Nachfolger traten abermals kurzfristige Hautblutungen und ein Thrombozytensturz auf. Danach blieb der Patient bei strenger Vermeidung einer Exposition gegenüber HCH beschwerdefrei. Bei einer Nachuntersuchung anläßlich einer Begutachtung im Institut für Berufskrankheiten im April 1959 war das Blutbild unauffällig. Die Thrombozytenzahl betrug 150000/µl. Blutungszeit, Gerinnungszeit, Prothrombinzeit ergaben normale Werte. Die Megakaryozyten im Mark waren quantitativ und qualitativ regelrecht, die Erythro- und Granulozytopoese zeigten normale Zellverhältnisse.

Wesentlich an dieser Beobachtung sind hinsichtlich der Insektizide die ausschließliche Exposition gegenüber HCH und die Hinweise auf eine allergische Genese der Thrombozytopenie.

Beobachtung 2 J. W., geb. 1900. Der 58jährige Zimmermann erkrankte im Herbst 1958 mit Appetitlosigkeit, Schwäche und Atemnot. Mitte Oktober 1938 wurde er arbeitsunfähig. Im folgenden Frühsommer ist er erstmals stationär behandelt worden. Dabei wurden ein schwerer Leberschaden und eine Panmyelophthise festgestellt. Im

Tabelle II (Fortsetzung)

Thrombocytopenie Mittel allein	Thrombocytopenie mit anderen Mitteln toxisch	Leukozytopenie Agranulozytose Mittel allein	Leukozytopenie Agranulozytose mit anderen Arzneimitteln unverd.	Leukozytopenie Agranulozytose mit anderen Arzneimitteln toxisch	Erythrozytäre Hypoplasie ohne Panzytopenie mit anderen Arzneimitteln unverdächt.	Erythrozytäre Hypoplasie ohne Panzytopenie mit anderen Arzneimitteln toxisch
	1		2			
2	1		5	1	1	
		2	1		1	1

Die Panmyelophthisen werden im anglo-amerikanischen Schrifttum nach EHRLICH meistens als aplastische Anämien bezeichnet.

Februar 1960 erfolgte eine Begutachtung im Institut für Berufskrankheiten, da inzwischen die Frage aufgetaucht war, ob die Erkrankung auf berufliche Einwirkungen zurückzuführen sei. Als Zimmermann hatte er in den Zeiten vom September 1955 bis Mai 1956 und vom Januar 1958 bis zu seiner Arbeitsunfähigkeit am 19. Oktober 1958 bei Dachstuhlsanierungen in großem Umfang das Holzschutzmittel Hylotox® verwendet. Das Mittel wurde von ihm ohne Arbeitsschutzvorkehrungen, insbesondere ohne Atemschutz, täglich bis zu 6 h lang mittels Handspritzen überwiegend in abgeschlossenen Dachstühlen ausgebracht.

Die Untersuchungen ergaben 1960 eine therapieresistente Anämie bei Serumeisenwerten im oberen Normbereich. Die Retikulozytenzahlen lagen zwischen 2 und 12‰. Das weiße Blutbild zeigte Leukozytenzahlen zwischen 4000 und 5000/µl und 15 bis 40% Granulozyten im Differentialblutbild. Die Thrombozytenwerte lagen um 100000/µl, der Coombs-Test war negativ. Wiederholte Sternalmarkausstriche zeigten eine megaloblastoide Erythrozytopoese. In der Granulozytopoese fiel lediglich eine Linksverschiebung mit Vermehrung der Promyelozyten auf. Insgesamt sprach das Bild für eine Reifungsstörung der Erythro- und Granulozytopoese. Die Anämie konnte nur durch Bluttransfusionen beeinflußt werden. Der untere Leberrand war 2 Querfinger unter dem rechten Rippenbogen tastbar. Konventionelle Leberproben waren bis auf eine gelegentlich pathologische Thymolprobe im Normbereich. Prontosilprobe, Elektrophorese sowie Transaminasen und Phosphatasen zeigten keine Auffälligkeiten. Eine Laparoskopie oder Leberpunktion hatte der Patient damals verweigert.

Im Frühjahr 1966 wurde der Patient auswärts erneut stationär wegen unveränderter therapieresistenter Anämie und Leukozytopenie behandelt. Bei dieser Untersuchung waren auch die Thrombozytenwerte bis auf 12500/µl abgefallen. Im Sternalmark fand sich wiederum eine Regenerationsstörung. Die Leberbiopsie ergab eine erhebliche chronische und vernarbende Hepatitis mit schwerer Siderose.

Das verwendete Holzschutzmittel enthielt DDT und HCH. Aus ähnlichen Einsätzen ist bekannt, daß dabei bis zu 100 l des Mittels täglich verbraucht werden. Eine ursächliche Bedeutung der chlorierten Kohlenwasserstoffe, besonders des HCH, ist in diesem Fall um so eher anzunehmen, als gleichzeitig ein chronischer Leberschaden vorlag, der sich zwar 7 Jahre später bei einer Biopsie als ein chronisch vernarbender entzündlicher Prozeß darstellte, dessen Ursprung jedoch sehr leicht eine toxische Leberschädigung

in den Jahren 1958/59 gewesen sein kann. SCHÜTTMANN (25) hat über einen in gleicher Weise gegenüber *Hylotox®* exponierten Zimmermann berichtet, bei dem sich eine bioptisch gesicherte toxische Fettleber entwickelte, deren Übergang in ein reaktiv entzündliches Stadium durch rechtzeitige und intensive Therapie verhindert werden konnte. Das gleichzeitige Auftreten einer Knochenmark- und einer Leberschädigung bei dem Patienten W. unter der Einwirkung eines sowohl die Leber wie auch die Hämatopoese angreifenden Arbeitsstoffes kann als Indiz für dessen ursächliche Bedeutung angesehen werden.

Beobachtung 3, E. V., geb. 1913. Bei dem 47jährigen Gärtner traten Ende 1960 allgemeine Mattigkeit, Schweißausbrüche und Herzbeschwerden auf. Nach einigen Monaten stellten sich abendliche Temperaturen zwischen 38 und 39 °C ein. Im Juli 1961 erfolgte in unverändertem Allgemeinzustand stationäre Aufnahme in einer HNO-Klinik wegen Tonsillitis, Zahnfleischentzündung und -blutung. Nach Anfertigung eines Blutbildes wurde der Patient wegen Agranulozytose in eine innere Klinik verlegt. Bei der klinischen Untersuchung fanden sich außer der Tonsillitis und Gingivitis Petechien an der Uvula und dem weichen Gaumen. Die Leber war bei der Aufnahme wie auch meist während der stationären Beobachtung 1 bis 1½ Querfinger unter dem rechten Rippenbogen tastbar, die Milz konnte nur zeitweise getastet werden. Das Blutbild zeigte bei leichter Anämie und etwas niedrigen Thrombozytenzahlen eine ausgeprägte Agranulozytose mit 1% Stabkernigen und 2% Segmentkernigen bei 2300 Leukozyten/µl. Der Sternalmarkausstrich war zellarm mit besonderer Reduzierung der Granulozytopoese und nur einigen Megakaryozyten. Der direkte und indirekte Coombs-Test verlief negativ.

Da der Patient seit 1948 bis zum Beginn der stationären Behandlung 1961 als Gärtner tätig war und besonders im Frühjahr und Herbst gerade der letzten Jahre intensiven Umgang mit HCH-haltigen Insektiziden hatte, mußte der dringende Verdacht auf eine insektizidbedingte Markschädigung entstehen. Nach der ärztlichen Meldung über eine Berufskrankheit wurde durch den Betrieb die starke Exposition besonders auch gegenüber HCH-haltigen Präparaten bestätigt. Es kam deshalb auch einer Begutachtung zur Anerkennung der Erkrankung als Berufskrankheit durch Schädlingsbekämpfungsmittel.

Am 10. September 1962 starb der Patient unter den Zeichen eines Herz-Kreislauf-Versagens. Die Sektion ergab als Grundleiden eine Knochenmarkinsuffizienz. Daneben fanden sich eine allgemeine Arteriosklerose und eine fortschreitende postnekrotische Leberzirrhose mit gemischttropfiger Verfettung der Regenerate.

Auch bei dieser Beobachtung ist die mit Verfettung einhergehende Leberzirrhose als besonderer Ausdruck einer toxischen Schädigung durch HCH anzusehen, zumal andere ursächliche Faktoren nicht nachweisbar waren. Bemerkenswert ist der Beginn der Erkrankung nach der Schädlingsbekämpfungsaktion im Herbst.

Beobachtung 4, G. J., geb. 1934. Im Herbst 1962 kam es bei dem 28jährigen Feldbaubrigadier wiederholt zu Episoden von Zahnfleischblutungen und Schwächezuständen. Im Frühjahr 1963 traten die gleichen Erscheinungen erneut auf, weshalb der Patient vom 4. Juni bis 16. Juli 1963 in einem Kreiskrankenhaus behandelt wurde.

Es bestanden eine Granulozytopenie (2300 Leukozyten/µl bei 76% Lymphozyten, 20% Segment- und 4% Stabkernigen) und Anämie (Hb 42%, Fl. 1,23). Da unter Bluttransfusionen und medikamentöser Behandlung keine Besserung auftrat, wurde der Patient an eine Universitätsklinik überwiesen, wo er bis zum 7. Dezember 1963 stationär war. Bei der Aufnahme war der Rumpel-Leede-Versuch stark positiv. Leber- und Milzvergrößerung bestand nicht. Im Blutbild fand sich eine Panzytopenie (3,08 Mill. per µl Erythrozyten bei 9,9 g% Hämoglobin, 2200/µl Leukozyten bei 50% Lymphozyten, 8000 µl Thrombozyten). Im Sternalmark wurde eine Reduzierung der Granulo- und Thrombozytopoese bei ausgeprägter lymphoretikulärer Begleitreaktion festgestellt. Da der zeitweilig direkt positive Bromelintest Wärme-Auto-Antikörper gegen Erythrozyten nachwies, wurde eine autoimmunologische Natur der Panzytopenie diskutiert. Bluttransfusionen wurden erforderlich. Gegen Ende der Behandlung mit hohen Prednisongaben stabilisierte sich der Befund bei Hämoglobinwerten zwischen 7,9 g% und 9,7 g%, 3–6000/µl Leukozyten und 10000/µl Thrombozyten. Zu diesem Zeitpunkt war der Bromelintest wiederholt negativ.

Während der sich anschließenden ambulanten Behandlung entstand der Verdacht, daß die hämatologische Erkrankung durch Pflanzenschutzmittel aus der Reihe der Halogenkohlenwasserstoffe verursacht sein könnte. Aufgrund einer Meldung über eine Berufskrankheit wurde der Patient im Juni 1965 im Institut für Berufskrankheiten begutachtet. Damals arbeitete er bereits wieder im Feldbau unter Vermeidung jeglichen Kontakts mit Pestiziden. Die hämatologische Untersuchung ergab im Sternalmark eine mäßige Hemmung der Erythrozytopoese, und im Blut lag eine Thrombozytopenie von 50000/µl vor. Das periphere rote und weiße Blutbild war unauffällig (Hb 13,8g%, 5,0 Mill./µl Erythrozyten, 5200/µl Leukozyten bei 3% Eosinophilen, 58% Segmentkernigen, 34% Lymphozyten und 5% Monozyten). Der Coombs-Test war direkt und indirekt negativ. Hinweise für eine Leberschädigung ergaben sich nicht. Der Patient war bis auf Herzklopfen bei Anstrengung beschwerdefrei. Eine Blutungsneigung bestand nicht mehr. Die Berufsanamnese ergab, daß der Patient seit 1960 als Feldbaubrigadier arbeitete. In dieser Eigenschaft war er stets für die Schädlingsbekämpfung verantwortlich. Verwaltung des Giftlagers, Transport der Präparate, Ansetzen der Lösungen und Auffüllen der Geräte wurden von ihm persönlich durchgeführt, ebenso das Ausbringen der Mittel. Die Spritz- und Stäubearbeiten begannen durchschnittlich Ende April, erstreckten sich mit Unterbrechung bis zum Herbst und betrugen monatlich etwa 3–4 volle Arbeitstage. Anfangs wurde mit Hand- und später mit Pferdespritzgeräten gearbeitet. Der Patient kam mit fast allen einschlägigen Pestiziden in Kontakt. Bei den Feldbauarbeiten handelte es sich um HCH-, DDT- und auch Phosphorsäureesterpräparate.

Nach Kenntnis der Schädigungsmöglichkeiten der Hämatopoese durch HCH mußte auch bei dieser Beobachtung einer Knochenmarkschädigung eine Berufskrankheit angenommen werden. Beachtenswert erscheint der zeitliche Zusammenhang. Die ersten Symptome traten im Herbst 1962 nach den Herbsteinsätzen auf. Sie klangen im Laufe des Winters ab, setzten aber im Frühjahr nach erneuter HCH-Exposition beim Stäuben wieder stürmisch ein und veranlaßten Krankenhauseinweisung mit Aufdeckung der Blutkrankheit.

Beobachtung 5 G. Sch., geb. 1928. Im April 1964 erkrankte der 36jährige Pflanzenschutzwart mit Abgeschlagenheit, Schwindelgefühl und häufigen Kopfschmerzen. Es fiel eine zunehmende Blässe auf. Im November erfolgte stationäre Einweisung in ein

Bezirkskrankenhaus, wo er wegen Panzytopenie mit Blut- und Erythrozytentransfusionen, Prednison und Antibiotika behandelt wurde. Anfang 1965 erfolgte zur Klärung der Diagnose Überweisung an die I. Medizinische Klinik der Charité. Die hämatologischen Untersuchungen ergaben damals: Hb. 9,9 g% Ery 3,29 Mill./µl, Hbe 30pg, Hämatokrit 26 Vol.% Leuko. 1450/µl, Meta. 1% Stab. 3% Segm. 23%, Lympho. 67%, Mono. 9% Retikulozyten rel. 22‰, abs. 72 380/µl, Thrombozyten 25 000/µl, Blutungszeit länger als 15 Gerinnungszeit Beginn 10' Ende 11 40" Rumpel-Leede-Versuch + Kneifversuch + Im Sternalpunktat vom 10. Februar 1965 wurde im Gegensatz zu späteren Untersuchungen eine starke Hyperplasie der Erythrozytopoese gefunden, die als Zeichen der Knochenmarkschädigung auch in qualitativer Hinsicht erhebliche Normabweichungen aufwies mit zahlreichen pathologischen Mitosen und ausgeprägter Kernlappungstendenz mit vielen kleeblattförmigen Figuren. Auch die vermehrten Promyelozyten zeigten auffällige morphologische Veränderungen. Die Megakaryozyten waren unauffällig. Die alkalische Granulozytenphosphatase hatte mit 296 eine erhöhte Aktivzahl. Der direkte und indirekte Coombs-Test war negativ. Im Verlaufe der nächsten Monate bestätigte sich bei wiederholten stationären Behandlungen und ambulanten Kontrollen die Verdachtsdiagnose einer Panmyelophthise. Bei einer Sternalpunktion am 18. Mai 1965 konnte nur extrem zellarmes Fettmark erhalten werden. Bei der dritten Punktion bestand das gewonnene Gewebe zum großen Teil aus Faser- und Fettmark. In diesen Anteilen lagen lediglich Plasmazellen, Lymphozyten und Gewebsmastzellen vor. Daneben gab es Abschnitte mit stärkerem Zellreichtum. Es fehlten jedoch die Zellen der Granulo- und Thrombozytopoese weitgehend. Diese zellreichen Abschnitte bestanden vorwiegend aus Zellen der Erythrozytopoese, wobei erhebliche Atypien, offensichtlich mit Mitosestörungen, vorhanden waren. Im peripheren Blut war jetzt eine Panzytopenie stark ausgeprägt: Hb. 8,8 g% Ery 2,90 Mill./µl, Hbe 31pg, Leuko. 1450/µl, Thrombozyten 7000/µl, Retl. rel. 2‰ abs. 5900/µl. Es kam zu häufigem und heftigem Nasenbluten. Als weitere Zeichen der starken hämorrhagischen Diathese bestanden an der Haut Hämatome und Petechien. Am 7. Februar 1966 starb der Patient im Heimatkrankenhaus. Die Sektion bestätigte die klinische Diagnose einer Panmyelophthise.

Da der Patient seit 1960 bis zum Beginn seiner Erkrankung also über 4 Jahre, als Pflanzenschutzwart tätig war, wurde schon frühzeitig an eine Knochenmarkschädigung durch HCH gedacht. Eine Nachfrage bei der zuständigen Pflanzenschutzstelle ergab, daß der Patient am häufigsten mit HCH- und DDT-Mitteln und deren Kombinationen gearbeitet hat. Aufgrund einer ärztlichen und betrieblichen Meldung über eine Berufskrankheit kam es zur Begutachtung, und die Erkrankung wurde als Berufskrankheit anerkannt. Wesentlich hinsichtlich der HCH-Schädigung sind bei dieser Beobachtung auch die Normabweichungen der Erythroblasten im Knochenmarkausstrich mit zahlreichen pathologischen Mitosen und ausgeprägter Kernlappungstendenz mit sogenannten Kleeblattfiguren. Diese Kernformen finden sich regelmäßig bei toxischen Markschäden durch Mitosegifte wie beispielsweise Arsen und Colchicin. Der Nachweis gerade dieser Kernveränderungen ließe sich im Sinne einer toxischen Einwirkung werten.

Beobachtung 6. W. H., geb. 1899: Ende Mai 1963 kam es bei dem 65jährigen Gärtner zu mäßigen Hämoptysen. Es bestanden Atemnot, Schwindelanfälle und Mattigkeit. In den nächsten Tagen traten diffuse Petechien und Nasenbluten auf. Daraufhin erfolgte stationäre Einweisung. Bei der Aufnahme war der Patient sehr blaß, die Schleimhäute zeigten eine schlechte Durchblutung. Der ganze Körper war mit Petechien bedeckt. Es bestanden Schleimhautblutungen unter der Zunge und an der Wangenschleimhaut. Leber und Milz waren geringgradig vergrößert. Die Achillessehnenreflexe konnten nicht sicher ausgelöst werden, der Patellarsehnenreflex war rechts lebhafter als links. Außerdem fiel eine Anisocorie zugunsten rechts auf. Die hämatologischen Laboratoriumsuntersuchungen ergaben die Befunde einer Panmyelophthise: Ery 2,02 Mill./µl, Hb 5,79 g%, Hb_E 28,7 pg, Leuko. 2000/µl, Stab. 3%, Segm. 9%, Lympho. 87%, Mono. 1%, Thrombozyten 40400/µl (20‰). Blutungszeit 9', Gerinnungszeit 7' 4". Bei der Sternalpunktion wurde nur wenig, nicht verwertbares Material gewonnen. Der direkte und indirekte Coombs-Test war negativ. Am 3. Tag der stationären Behandlung starb der Patient unter den Zeichen einer zentralen Regulationsstörung. Die Sektion ergab in Übereinstimmung mit der klinischen Diagnose eine ausgeprägte Knochenmarksinsuffizienz und als direkte Todesursache eine zentrale Regulationsstörung bei mehreren Blutungen im Bereich des Parietal- und Kleinhirns sowie eine frauenhandtellergroße Subarachnoidalblutung. Außerdem wurden als Ausdruck der hämorrhagischen Diathese zahlreiche petechiale Haut- und Schleimhautblutungen gefunden. In der Leber bestand eine fleckförmige gemischttropfige Verfettung.

Als mögliche Ursache für die Panmyelophthise kam nur der Kontakt gegenüber HCH-haltigen Insektiziden in Frage, dem der Patient als Gärtner bei der Schädlingsbekämpfung fast laufend ausgesetzt war. Andere auslösende Faktoren ließen sich nicht nachweisen. Auch bei dieser Beobachtung waren die histologisch gefundenen Leberveränderungen durch eine HCH-Schädigung erklärbar.

Diskussion

Die von uns mitgeteilten Beobachtungen hämatologischer Schäden bestätigen die im internationalen Schrifttum veröffentlichten Feststellungen, wonach im Umgang mit Insektiziden aus der Reihe der chlorierten Kohlenwasserstoffe, insbesondere durch HCH, nicht selten Störungen der Blutbildung auftreten. Offenbar kommen Erkrankungen besonders dort vor, wo die Anwendung der Mittel in großem Umfang erfolgt. Das HCH eignet sich wegen des relativ hohen Dampfdrucks sehr gut zur Anwendung in Vernebelungsgeräten. Der amerikanische Markt hatte eine große Zahl elektrischer Vernebler angeboten, die in Haushalten und im Gewerbe in geschlossenen Räumen, oft im Dauerbetrieb benutzt wurden. Die Häufung hämatologischer Schäden durch HCH-Einwirkung ist besonders in den USA auf diese äußerst intensive Exposition bezogen worden. Die Zusammenhänge wurden bald erkannt. Bereits anfangs der fünfziger Jahre wurde

vor der Anwendung der Vernebler von HCH dringend gewarnt. Aber auch in anderen Staaten ist die schädigende Wirkung des HCH auf die Blutbildung geläufig. So führt Trotskij (28) in der UdSSR in einer Übersicht über berufsbedingte Anämien in der Gruppe der hypo- oder aplastischen Knochenmarkschäden neben energiereichen Strahlen und Benzol ausdrücklich das Lindan an. Unsere Beobachtungen lassen erkennen, daß bei Aufmerksamkeit auch in Mitteleuropa entsprechende Krankheitsfälle durch die Einwirkung von HCH zu erkennen sind.

Ähnlich wie in anderen Ländern kann auch von uns aufgrund dieser kasuistischen Beobachtungen keine Aussage über die tatsächliche Häufigkeit derartiger Erkrankungen abgegeben werden, da einmal die Zahl der Gefährdeten und die Intensität der Exposition unbekannt sind, zum anderen aber bislang sicher bei weitem nicht alle Fälle von Anämie, Granulozytopenie, Thrombozytopenie und Panmyelophthise unter der Einwirkung von HCH als solche erkannt und gemeldet wurden. Wir wollen mit dem Bericht dazu beitragen, daß bei Knochenmarkschäden in Zukunft mehr als bisher bei der Suche nach möglichen exogenen kausalen Faktoren auch an die Insektizide, speziell das HCH, gedacht wird. Dabei muß vorerst noch offen gelassen werden, welcher Pathomechanismus den einzelnen Fällen zugrunde liegt.

In erster Linie muß wohl eine primär toxische und eine allergische Ätiologie erörtert werden. Obwohl verschiedene Autoren (1, 16, 17) bei der Diskussion ihrer unter HCH-Einwirkung geschehenen hämatologischen Erkrankungen einen immunologischen Mechanismus erwogen haben, ist ihnen der Nachweis eines solchen nicht gelungen. Der Thrombozyten- und Granulozytenantikörpernachweis hat sich leider noch nicht für die Routinepraxis der Allergendiagnostik bewährt, zumal die Agglutinine einen flüchtigen Charakter haben und nicht in allen Fällen nachweisbar sind. Mirscher (18) konnte sie nur in 30% der Fälle von Pyramidon-Agranulozytose nachweisen. Lediglich Muirhead et al. (21) haben bei einem Landwirt mit Kontakt zu Heptachlor® und Dieldrin®, also dem HCH ähnlicher chlorierter Kohlenwasserstoffverbindungen, eine erworbene hämolytische Anämie beobachtet, bei der sie mittels stark positiven Coombstestes nach Zubereitungen der entsprechenden Insektizide eine allergische Natur der hämolytischen Reaktion wahrscheinlich machen konnten. Außerdem kam es zu einem Rückfall der Erkrankung als für

kurze Zeit eine erneute Exposition gegen Pflanzenschutzmittel stattfand. Unter unseren Erkrankungsfällen spricht die Beobachtung mit positiven Thrombozytenagglutinationstesten, wie bereits GALLS (11) anderweitig erörtert hat, für die Möglichkeit einer allergischen Genese der Thrombozytopenie.

Auch die Möglichkeit der allergischen Reaktion vom sogenannten Turberkulin- oder Spättyp durch selbständige Antikörper muß in Betracht gezogen werden. Nach RAJKA (23) kann zum Beispiel ein Arzneimittel, das an den Eintrittspforten (Atemorgane, Magen-Darm-Trakt) eine Frühreaktion auslöst, an anderen Organen eine Spätreaktion zur Folge haben, wenn es, wahrscheinlich mit Autoantikörper kombiniert, zur Sensibilisierung dieser Organe führt.

Andererseits liegen Untersuchungsergebnisse vor, die mehr an einen primär toxischen Wirkungsmechanismus denken lassen. FERRERO und DODSON (9) haben mitgeteilt, daß bei Ratten, die mit einer einmaligen HCH Dosis vergiftet wurden, sowohl unmittelbare Knochenmarkpräparationen als auch Leukozytenkulturen eine Depression der Mitoserate erkennen ließen. Danach wäre mit einer echten zytotoxischen Wirksamkeit des HCH zu rechnen, wie sie bei unserer Beobachtung 5 in Betracht kommt. Auch angeborene oder erworbene Enzymschäden könnten, ähnlich dem Glukose-6-Phosphat Dehydrogenase-Mangel der Erythrozyten als Ursache einer hämolytischen Anämie nach Phenazetin und zahlreichen anderen Substanzen ursächlich für die HCH Einwirkung von Bedeutung sein.

In den Listen der bei immunologischen Zytopenien als Antigene nachgewiesenen Stoffe wurde HCH bislang nicht angeführt (7, 18). Möglicherweise handelt es sich beim HCH um einen Stoff, dem sowohl eine toxische als auch eine allergisierende Wirksamkeit eigen ist. Eine solche doppelte Wirkung ist auch für andere Substanzen diskutiert worden. So weist NIEWEG (22) darauf hin, daß das gleiche Pharmakon z. B. Chinin einmal nachweislich allergische Blutschäden verursacht, zum anderen aber auch hämatologische Schäden hervorruft, bei denen ein immunologischer Mechanismus nicht festzustellen, sondern vielmehr eine enzymblockierende toxische Wirkung anzunehmen ist. Auch die außerordentlich unterschiedliche Wirksamkeit der Therapie bei den HCH Panmyelophthisen könnte durch verschiedene Pathomechanismen der Knochenmarkschädigung bedingt sein.

Gründliche immunologische, toxikologische, zytochemische und enzymkinetische Untersuchungen bei künftigen Erkrankungen werden die Frage der Pathogenese weiter abklären müssen. Schon aus diesem Grunde sollten einschlägige Fälle an dafür geeignete Kliniken überwiesen werden. Ferner darf nicht vergessen werden, daß hämatologische Erkrankungen unter der beruflichen Einwirkung von Pestiziden als Berufskrankheitsverdacht meldepflichtig sind. Die dadurch ermöglichte vollständige Erfassung der Fälle wird erlauben, zukünftig verbindlichere Aussagen über die tatsächliche Häufigkeit hämatologischer Krankheitsbilder durch Einwirkung dieser Stoffe abzugeben.

Eine Klärung dieser Fragen ist schließlich nicht nur für die beruflich gefährdeten Personen bedeutsam, sondern auch für die gesamte Bevölkerung. Mit zunehmender Anwendung der Insektizide aus der Reihe der chlorierten Kohlenwasserstoffverbindungen ist infolge Rückstandsbildung und Ablagerung in den verschiedensten pflanzlichen und tierischen Lebensmitteln und durch Anwendung der Mittel in Haus und Garten mit einer allmählich wachsenden, wenn auch zunächst noch geringfügigen Exposition aller Menschen zu rechnen.

Herrn Chefarzt Dr. med. Zeiss, Innere Klinik des Bezirkskrankenhauses Wismar, Herrn Prosektor Dr. med. Möbius, Pathologisches Institut des Bezirkskrankenhauses Schwerin und Herrn Prosektor Dr. med. Wolf, Pathologisches Institut des Bezirkskrankenhauses Cottbus, danken wir sehr für die Überlassung von Krankengeschichten bzw. Sektionsprotokollen.

Zusammenfassung

6 eigene Beobachtungen von Knochenmarkschäden nach beruflicher HCH-Exposition zeigen, daß auch in Mitteleuropa mit derartigen Krankheitsbildern zu rechnen ist. Es wird auf die Notwendigkeit hingewiesen, in allen Fällen von Störungen der Blutbildung bei der Ursachenforschung auch nach einer möglichen Einwirkung von Pestiziden, vor allem HCH, zu fahnden. Bei der Diskussion der Vorstellungen zur Natur der Schadwirkungen wird auf einen möglichen allergischen und toxischen Wirkungsmechanismus eingegangen.

Summary

Bone marrow damage observed in 6 cases after professional exposure to hexachlorcyclohexane (HCH) shows that this condition may also be met with in Central Europe. Attention is drawn to the need in all cases of haemopoietic disorders, in searching for the causes, to bear in mind the possibility of poisoning with pesticides, and especially HCH. In discussing the nature of the pathogenic mechanisms, allergic and toxic manifestations are considered.

Résumé

6 observations personnelles d'effets nocifs de l'exposition professionnelle au gamma-hexachlorcyclohexane sur la moelle osseuse démontrent qu'il faut compter avec l'apparition possible de tels tableaux cliniques en Europe centrale. Il est insisté sur la nécessité de rechercher dans tous les cas d'une perturbation de l'hématopoïèse l'effet éventuel d'un pesticide (insecticide) surtout d'HCH. Dans la discussion sur la nature de l'effet nocif, un mécanisme allergique ou toxique est pris en considération.

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AB0 Blood Group Agglutinins in Saliva

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In 1952 Putkonen (15) reported on the frequency of AB0 blood group agglutinins in saliva. Using simple agglutination tests, he detected agglutinins in saliva from 103 of 728 donors (14%). Agglutinins were detected in 53 of 172 salivas from donors of group 0 (31%), 33 of 317 salivas from donors of group A (9.5%) and 17 of 136 salivas from donors of group B (12.5%).

Following the work of Miyakoshi (11) who proposed that the presence of AB0 agglutinins in saliva is under the control of a single gene, with absence of agglutinins dominant to their presence, Furuhata *et al.* (7) recorded histograms for the titres of anti A in B and 0 salivas, and anti B in A and 0 salivas and drew up tables comparing the observed numbers of children and their phenotypes from matings in which the parents were classified as to whether or not their salivas possessed agglutinins. The data were considered to be consistent with the expectations based on Miyakoshi's model. However the data did not differentiate between the frequencies of anti-A in B and 0 salivas or anti B in A and 0 salivas. This inadequacy was criticized by Prokop (14) who found, as had Putkonen (15) that agglutinins are detectable in salivas of 0 persons more frequently than in salivas from A_1, A_2 or B persons. He found, also anti-B in a higher proportion of salivas from A_2 individuals than in those from A_1 individuals.

Prokop (14) considered that, due to the significantly different frequencies of individuals in the different blood groups possessing detectable salivary agglutinins the simple mode of inheritance proposed by Miyakoshi (11) is inadequate to explain the occurrence of AB0 agglutinins in saliva, and suggested that the higher proportion of 0 salivas with anti-B than A salivas is a reflection of the lower molecular weight of the anti B agglutinins in 0 sera.

WILSON and GREEN (17) too found agglutinins in a higher proportion of salivas from group 0 persons than in those from A or B individuals, and recorded pedigrees which show that, if secretion of AB0 agglutinins is under direct genetical control, it can not be either a simple autosomal dominant or a sex-linked recessive character However these authors felt that the ability to secrete salivary agglutinins is genetically influenced.

A study of the presence of AB0 agglutinins in saliva was undertaken as part of a project to investigate a proposed conceptual scheme for infertility in some humans, based on AB0 blood group incompatibility (1) The scheme envisaged a reaction between AB0 blood group antigens on spermatozoa and the appropriate antibodies in the mucous secretion of the uterine cervix.

GERSHOWITZ *et al.* (8) had reported the presence of AB0 agglutinins in mucus from the uterine cervix but the factors controlling their presence were not known. Since the presence of AB0 antigens in one mucous body secretion can be inferred by detecting them in another it was hoped that a similar relationship existed with AB0 agglutinins. Since cervical mucus is not easily obtainable, and only small quantities can be obtained in any case, the factors controlling the presence of AB0 agglutins in mucous secretions could be investigated most conveniently with saliva. Comparative studies on saliva and cervical mucus from the same individuals were proposed to determine whether the results from studies on saliva could be applied to cervical mucus.

Materials and Methods

Most of the saliva specimens were obtained from university students. These were obtained in sterile 1 oz. wide-mouthed McCartney bottles either mid-morning or mid-afternoon. At the same time, each student provided a blood specimen from finger-prick for grouping, and recorded his name, sex, age and country of birth on sheet next to the number appropriate to his specimen.

The saliva specimens were deep-frozen as soon as possible after collection. On removing saliva specimens from deep-freeze it was found that, on thawing, the materials responsible for the viscosity of saliva had aggregated and were readily precipitated by centrifugation. The supernatants were clear non-viscous and were easy to handle, unlike the neat saliva. Another technique satisfactory for removing the viscosity of saliva without affecting AB0 antigens or agglutinins in it was suggested by Mr. G. Vos. Equal volumes of saliva and ether are shaken for 10 min and the mixture is centrifuged for 5 min at 3,000 rpm. This produces three layers, a top layer of ether semi-solid middle layer, and a lower layer of clear saliva which is non-viscous and is easily handled. Although tests showed that this technique did not affect antigen or agglutinin titres, it was not used in any of the work reported here.

All saliva specimens were tested against 2% suspensions of A_1, A_2, B and 0 cells because the blood group of any donor was not related to the agglutinins detected in his saliva until after all results had been obtained. The anti-A titres reported are those for reactions with A_1 cells.

The two specimens of cervical mucus tested were provided by Dr F WELLER. Each specimen was collected on sterile, cotton-wool swab. Identical swabs had been tested and shown not to possess blood group specificity Since mucus could not be expressed from the swabs with Spencer Wells forceps, eight drops of saline were added to each, and they were then allowed to stand for 15 min. Clear non-viscous liquid was obtained from the swabs by putting the tip of Pasteur pipette against them, and sucking. This liquid was used as the cervical mucus specimen for testing

Results

Using saliva specimens taken at irregular intervals over a period of two years from members of the laboratory staff and of my own family and testing for the presence of AB0 agglutinins, it has been found that all specimens from the one individual recorded the same result throughout, i. e. those persons whose salivas contained agglutinins showed them at each testing and those persons whose salivas did not contain agglutinins did not show them at any of the testings. Further the titre of agglutinins in saliva specimens taken at the same time daily from four individuals possessing them (one A_1 one B and two 0's) proved to be stationary for the period of a month over which the salivas were tested All salivas were deep-frozen immediately after collection and were tested on the same day with the same 2% cell suspensions. The titres varied, at most, one doubling dilution from the respective modes. These results indicate that the secretion of AB0 agglutinins in saliva is a stable condition and are in agreement with the results of PROKOP (14) who found that, though immunization increased serum titres markedly there was little effect on salivary agglutinins, and are contrary to those of SCHOENBRUCKER and FLATOW (16) who reported variation in salivary agglutinin titre with time, and even daily variation.

Saliva specimens from university students were collected and tested during three separate periods approximately six months apart. To be able to test for heterogeneity between the results recorded for salivas in the separate periods, these results were mutually kept separate. The results were grouped according to blood group sex, period during which the saliva was tested and birthplace.

Tests of heterogeneity established that the blood group frequencies of European born and Australian-born students were

Table I

The presence of AB0 agglutinins in salivas.

Period of testing	0 Salivas anti-A only				A_2 Salivas anti-B		A_1 Salivas anti-B		B Salivas anti-A		Totals
	anti-A only	anti-B only	Anti-A and anti-B	none	anti-B	none	anti-B	none	anti-A	none	Totals
1	10	4	46	16	9	6	23	34	12	6	166
2	12	20	112	26	24	11	53	60	27	18	363
3	24	12	115	21	24	17	62	83	27	20	405
Totals	46	36	273	63	57	34	138	177	66	44	934

significantly different (2) But within individual blood groups, there was no significant heterogeneity for the proportions of individuals of different birthplaces who recorded salivary agglutinins and those who did not. (The numbers of Asian-born students were too few to test statistically.) Accordingly the data within individual blood groups were pooled Further tests showed that, within individual blood groups, there was no significant heterogeneity of the data between sexes, so the data for both sexes were pooled.

The grouped data are presented in Table I These show that there is no significant heterogeneity between the results recorded during the three separate periods during which the saliva specimens were collected and tested and, therefore, it seems that the method of testing is reliable and that over the whole period the sensitivity of testing did not alter

The frequency of anti-A in 0 salivas is significantly greater than in B salivas. ($\chi_1^2 = 27.73$ $P < 0.001$) as is the frequency of anti B in 0 salivas when compared with A_2 ($\chi_1^2 = 18.42$ $P < 0.001$) and A_1 salivas ($\chi_1^2 = 115.6$ $P < 0.001$) Of special interest is the comparison of the frequency of anti B in A_1 and A_2 salivas.

	Anti-B detected	Anti-B not detected	
A_1 salivas	138	177	315
A_2 salivas	57	34	91
	195	211	406
	$\chi_1 = 10.03$	$0.01 > P > 0.001$	

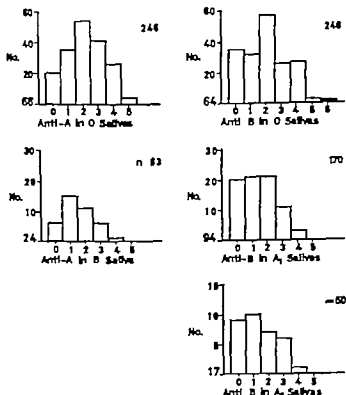


Fig 1 Log_2 titres of AB0 agglutinins in salivas from A_1, A_2, B and 0 individuals. The numbers of salivas from individuals of the respective blood groups in which agglutinins were not detected are indicated to the left of the y-axis.

These data show that anti-B is detectable in a higher proportion of group A_2 individuals than of group A_1 individuals.

The titres of agglutinins in 529 of the salivas were determined and are presented in Fig 1 It is noted that anti-A_1 was detected in the saliva of two individuals, one A_2 and one A_2B. Subsequent testing showed that these individuals possessed anti-A in the serum also.

During this study pedigrees were collected which contradict salivary agglutinin secretion being either a dominant or recessive character under the simple control of a single autosomal locus (Fig 2) and, since the population data do not show a significant difference between the frequencies of the phenotypes in males and females, control by a sex-linked locus is ruled out.

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Die Zeitfolge der DNS-Verdoppelung der Chromatinstrukturen in Interphasekernen kultivierter Leukozyten*

T. Büchner und R. A. Pfeiffer

Seit Heitz (14, 15) werden die stärker angefärbten Abschnitte der Prophasechromosomen als heterochromatisch, die schwächer angefärbten als euchromatisch bezeichnet. Auch im Interphasekern wird zwischen dichtem Heterochromatin und lockerem Euchromatin unterschieden. Was diesen Phänomenen zugrundeliegt, ist noch nicht aufgeklärt. Es werden Änderungen der Spiralstruktur (23) und der Hydratation (1) verantwortlich gemacht. Das Chromatinmuster der Zellkerne ist nicht konstant. Von Grundmann und Stein (9) wird auf Unterschiede zwischen verschiedenen Geweben besonders im Vergleich mit Karzinomzellen hingewiesen.

Auch in den durch Phytohämagglutinin transformierten Lymphozyten in der Kultur (20) tritt der Unterschied zwischen Eu- und Heterochromatin deutlich hervor. Heterochromatin wird in groben Schollen um die Nukleoli und diffus über den Kern verteilt gefunden. In diesen Zellen ist das Sexchromatin meist nicht sicher abzugrenzen.

Die beiden Chromatintypen unterscheiden sich im Zeitplan der DNS-Verdoppelung, wie die autoradiographische Markierung nach Einbau von H3-Thymidin zeigt. Es liegt deshalb nahe, das Chromatinmuster des Interphasekerns mit dem autoradiographischen Markierungsmuster der Chromosomen in der Metaphase zu vergleichen, denen ebenfalls eine untereinander verschiedene Zeit

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Herrn Prof. Dr. W. H. Hauss zum 60. Geburtstag

folge der Reduplikation eigen ist. LIMA DE FARIA *et al.* (18) ordnen das Heterochromatin besonders den akrozentrischen Chromosomen zu, die im Prophasekern heteropyknotisch erscheinen und in der Nachbarschaft der Nukleolen gefunden werden

Die Aufklärung der Beziehung zwischen interphasischem Chromatin und bestimmten, autoradiographisch gekennzeichneten Chromosomenabschnitten setzt die Kenntnis der Reihenfolge der DNS-Synthese an den Chromatinstrukturen voraus. Ist diese bekannt, dann lässt sich auch ein Zeitplan für den Ablauf der interphasischen Chromatinverdoppelung aufstellen.

Material und Methode

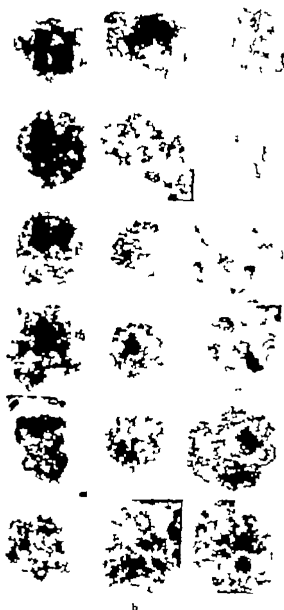
Die Untersuchung erfolgte an Zellen des peripheren Blutes einer gesunden Frau mit normalem Karyotyp. In einem Kulturmedium aus autologem Plasma und Medium 199 (1 3) 72 Stunden inkubiert, wandelten sich unter Einfluß von Phytohämagglutinin Lymphocyten in blastomatöse, teilungsfähige Zellen um.

30 min vor Beendigung einer Kultur wurde der spezifische DNS Vorläufer H3-Thymidin (1 µC/ml Medium, spez. Akt. 5 C/mM) allein, einer 2. Kultur H3 Thymidin 6½ Stunden vor der Präparation zusammen mit Colcemid zugesetzt. Nach Inkubation der abzentrifugierten Zellen in 1%iger Natrium-Zitratlösung wurde nach Carnoy I fixiert und die Zellen auf den Objektträgern ausgebreitet. Für die Autoradiographie wurde die Kernspuremulsion Ilford K2 bei 40 °C geschmolzen, 1 1 mit Wasser verdünnt und durch Eintauchen auf die Präparate gebracht. Nach einer Exposition von 14 Tagen wurde mit Metol-Hydrochinon-Entwickler 5 min entwickelt, mit wäßriger Natriumthiosulfatlösung 5 min fixiert und kurz gewässert. Die Präparate wurden 20 min lang mit gepufferter (pH 5 75) Giemsalösung gefärbt. Um die Morphologie der Zellen besser beurteilen zu können, wurden nach Photographie der markierten Zellen die Silberkörner mittels photographischem Abschwächer entfernt.

Ergebnisse

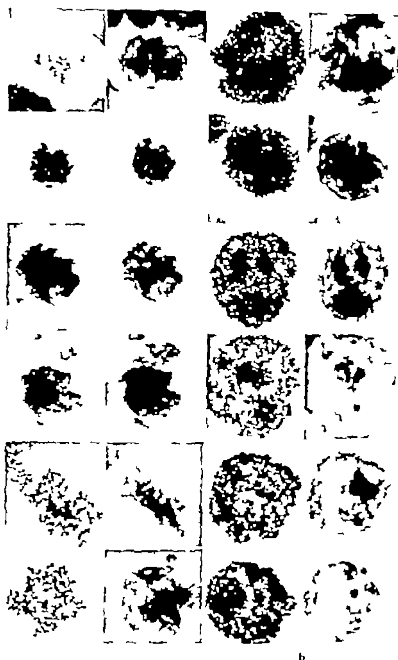
a) In Präparaten der Kultur nach 30 min Inkorporation von H3 Thymidin wurden 500 Zellkerne auf die Lagebeziehung von Eu und Heterochromatin zur autoradiographischen Markierung geprüft. Dabei konnten folgende Typen des Markierungsmusters unterschieden werden

Abb. 1 3 verschiedene autoradiographische Markierungsmuster der Zellkerne nach kurzzeitiger (30 min) Inkubation der Blutkultur mit H3 Thymidin. Der Markierungsgrad liegt bei allen Kernen in der gleichen Größenordnung Spalte enthält Zellkerne von bevorzugter Markierung des hellen Euchromatins, Spalte b solche mit ziemlich homogener Verteilung der Silberkörner ohne eine erkennbare Beziehung zu einem bestimmten Chromatintyp I Spalt sind die Kerne hauptsächlich über dunklen Heterochromatinblöcken und gar nicht oder kaum über dem Euchromatin markiert Die Spalten entsprechen der frühen S-Phase (einer längeren Z ischenphase (b und der abklingenden S-Phase (



b

1 14 / der Zellkerne zeigten eine ausschließliche oder überwiegende Markierung heller d. h. euchromatischer Kernbezirke mit Aussparung des Heterochromatins besonders der Nukleolen (Abb. Ia) 2. 76 der Kerne waren gleichmäßig markiert eine



b

Beziehung zu einem Chromatintyp war nicht zu erkennen (Abb. 1b) 3 In 10 der Zellkerne war das Heterochromatin deutlich bevorzugt markiert Abb 1c)



Abb. 3. Zellkern aus der Blutkultur nach 6½ Stunden Inkubation mit H3 Thymidin + Colcemid. Trotz schwacher Markierung liegen die Silberkörner vor allem über dem dunklen Heterochromatin. Dieses Muster fanden wir nur bei 2 von 100 Zellkernen. Die Ursache könnte eine außergewöhnlich lange G_2-Phase für diese Zellen sein.

b) In Präparaten der Kulturen, denen H3 Thymidin und Colcemid 6½ Stunden lang zugefügt war, wurden 1500 Zellkerne ausgewertet. Es fanden sich alle Grade der Markierung bis zur völligen Bedeckung des Kerns mit Silberkörnern. Zwischen der Zahl der Silberkörner und ihrer Lokalisation über dem Zellkern fanden sich folgende Beziehungen: In schwach markierten Kernen war fast nur Euchromatin markiert, während der größte Teil des Heterochromatins ausgespart war (Abb. 2a). Diese Anordnung lag in 98 von 100 Kernen mit weniger als 100 Silberkörnern (Grenze der Auszählbarkeit) vor. 2 Kerne waren dagegen bevorzugt über dem Heterochromatin markiert (Abb. 3). In einer Gruppe mittlerer Markierungsgrade (etwa 100 bis 300 Silberkörner) fanden sich Kerne mit diffuser Schwärzung ohne erkennbare Beziehung zu einem der beiden Chromatintypen. Eine dritte Gruppe von Kernen mit mittlerer bis starker Markierung zeigte eine intensive Markierung in Form von 1 bis 3 Verdichtungen. Nach Entfernen der Silberkörner waren diese Stellen als Heterochromatinblöcke zu erkennen (Abb. 2b). Das Euchromatin der Kerne in dieser Gruppe war mittelstark oder nur schwach markiert.

Abb. 2. Autoradiographisches Markierungsmuster in der Blutkultur einer gesunden Frau nach 6½stündiger Inkubation der Kultur mit H3-Thymidin + Colcemid. Die schwach markierten, in der frühen S-Phase erfaßten Kerne ( ) sind bevorzugt über hellen, euchromatischen Anteilen markiert, während das dunkle Heterochromatin, das in einigen Zellen dem Nukleolus assoziiert ist, von der Markierung weitgehend ausgespart bleibt. In den stark markierten, während der späten S-Phase oder der G_1 Phase erfaßten Zellkernen (b) ist besonders (z. T. Nukleolus-assoziiertes) Heterochromatin autoradiographisch markiert, während das hellere Euchromatin noch mehr oder weniger stark mitmarkiert ist. Die beiden Spalten a und b zeigen die Kerne jeweils vor und nach Entfernen der Silberkörner.

Diskussion

Die DNS-Synthese der eu- und heterochromatischen Abschnitte erfolgt nicht während der ganzen S-Phase gleichzeitig. Dieses Phänomen wurde bereits von LIMA DE FARIA (16) an Autosomen von rye grass gezeigt. In Zellen der Spermatopoese von Melanoplus konnte er nachweisen, daß das Heterochromatin gegenüber dem Euchromatin verzögert redupliziert. Eindrucksvoll ist dieses allozyklische Verhalten eines besonderen Typs von Heterochromatin, des Sexchromatins. ATKINS *et al.* (4) konnten z. B. an Fibroblasten in der Kultur feststellen, daß nach kurzfristiger H3-Thymidinaufnahme das Sexchromatin entweder selektiv markiert war oder ausgespart blieb. Zuvor hatte TAYLOR (25) an Zellen des chinesischen Hamsters Asynchronie kleiner Kernareale festgestellt, die in Form und Größe den heterochromatischen Anteilen des X- und Y-Chromosoms entsprachen.

Auch in menschlichen Lymphozyten in der Kultur unterscheiden sich Euchromatin und autosomales Heterochromatin in der Zeitfolge der Reduplikation (17). Nach kurzfristiger H3-Thymidinzugabe fand man Zellen mit selektiver Markierung und andere mit Aussparung der Heterochromatinblöcke.

ALTMANN *et al.* (2) sahen nach H3-Thymidinincorporation in Zellen der regenerierenden Rattenleber gelegentlich ausschließliche Markierung des Nucleolus. Sie folgerten daraus, daß die Synthese dort nicht gleichzeitig mit dem »Karyoplasma« stattfindet. Auch die elektronenmikroskopisch-autoradiographische Untersuchung an Zellen der Affennierenkultur (9) ließ sich im gleichen Sinne deuten. HARRIS (13) hatte auf Grund autoradiographischer Befunde an Bindegewebszellen vermutet, daß die DNS-Synthese am Nucleolus beginnen müsse. Das Markierungsmuster nach kurzer H3-Thymidin-Verfügungszeit (Pulsmarkierung) läßt allerdings nur die Asynchronie zwischen Eu- und Heterochromatin erkennen. Die Reihenfolge der Verdoppelung dieser Strukturen kann nur festgestellt werden, wenn eine ausreichend lange Verfügungszeit des H3-Thymidins von etwa $6^1/_2$ Stunden gewählt und durch gleichzeitige Zugabe von Colchicin der Abschluß der Mitosen markierter Zellen verhindert wird. Legt man die von CAVE (6) ermittelte Dauer der intermitotischen Phasen G_1, S und G_2 in Leukozytenkulturen zugrunde (S-Phase im Mittel 9,6 Stunden, G_2 3,5 Stunden), dann muß bei einer H3-Thymidinverfügungszeit von $6^1/_2$ Stunden ge-

schlossen werden, daß sich schwach markierte Zellkerne zum Z punkt der Präparation am Anfang der S-Phase und stark mark etwa im letzten Drittel der S-Phase oder der G_2 Phase befan Zellen, die kurz vor dem Abschluß der Synthese standen, als I Thymidin zugesetzt wurde, müssen innerhalb der 6½ Stunden die Mitose eingetreten sein und als markierte Metaphaseplatten ir Präparat vorliegen. Ihre Chromosomen sind nur auf den spätreu plizierenden Abschnitten markiert (Abb 4) Durch dieses semi quantitative Verfahren ist die zeitliche Orientierung innerhalb de S-Phase möglich (Abb 5) Für die Richtigkeit dieser Betrachtu spricht, daß die stark markierten, in der späten S-Phase erfaßte Zellen im Durchschnitt größer sind als die schwach markierter offenbar ein Ausdruck ihrer synthetischen Aktivität (Abb 2 Ob das Chromatin dieser Zellen während der Synthesephase einem ähnlichen Strukturwandel wie in den Zellen der Mäuseleber (3 19) unterworfen ist, wäre ergänzend zu prüfen

Chromatinverdichtungen, die durch Lage, Größe und auf fallend allozyklische Reduplikation als Sexchromatin anzuschen wären ließen sich in den von uns untersuchten Zellen nicht f st stellen, obwohl sich in den markierten Metaphasen deutlich spät reduplizierende X Chromosomen darstellten (Abb 4) RIBAS MUNDO (21) hatte bei einem Fall von X Isochromosom des langen Arms stark markierte Verdichtungen in kultivierten Lymphozyten gesehen, die offenbar zum gleichen Zeitpunkt wie die Nucleoli reduplizierten. Da die stark markierten Interphasekerne die bevor

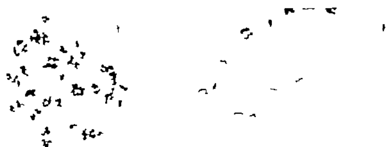


Abb 4 Typische Markierung der spätreduplizierenden Chromosomenabschnitte nach H3 Thymidineinbau in der späten S-Phase (Blutkultur 6) H3 Thymidin + Colcemid bei 9 Uhr sieht man das spätreduplizierende X-Chromosom, das über dem langen Arm noch besonders stark markiert ist, weniger über dem kurzen. Die hier markierten Chromosomenabschnitte würden heterochromatischen Anteilen des Interphasekerns (Abb. 2) entsprechen.

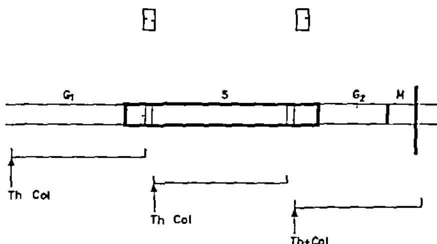


Abb. 5. Die langzeitige ($6\frac{1}{2}$ Stunden) Inkubation der Blutkultur mit H3-Thymidin + Colcemid gestattet eine zeitliche Orientierung innerhalb der S-Phase (9 Stunden), denn schwach markierte Zellkerne müssen sich bei der Präparation kurz nach Beginn ihrer DNS-Synthese befunden haben (links), während stark markierte etwa im letzten Drittel der S-Phase (Mitte) oder in der G_2-Phase ($3\frac{1}{2}$ Stunden) erfaßt wurden. Zellen nach Einbau von H3-Thymidin in der späten S-Phase sind nach den $6\frac{1}{2}$ Stunden Inkubationszeit bereits in die Mitose übergegangen und eher schwach markiert (rechts). Kurzzeitige H3-Thymidininkubation erlaubt keine zeitliche Orientierung, da die Silberkornzahl dann stets in der gleichen Größenordnung liegt (2 obere Kästchen). Die Dichte der schwarzen Punkte in diesem Schema symbolisiert den Grad der Aktivität einer Zelle durch H3-Thymidinaufnahme

zugte Markierung des Heterochromatins, die schwach markierten aber nur über dem Euchromatin Silberkörner zeigen, ist der Schluß berechtigt, daß die Synthese am Euchromatin beginnt und am Heterochromatin endet. Diese Folgerung teilen wir mit Ribas-Mundo (21), der jedoch bei seiner Versuchsanordnung (in den letzten 6 Stunden H3-Thymidin, in den letzten 2 Stunden Colcemid) damit rechnen mußte, daß ein Teil der schwächer markierten Zellen aus während der Thymidenzeit abgelaufenen Mitosen hervorgegangen ist. Aus den Ergebnissen von Cave (6) geht hervor, daß die Dauer der G2-Phase stark variiert; sie beträgt bei 7% der Zellen weniger als 2, sogar bei 35% weniger als 3 Stunden. Die Mitosedauer dieser Zellen ist bisher nicht bekannt. Nach Lebendbeobachtungen an verschiedenen Zellarten (Übersicht bei Grundmann 10) kann diese in einer Größenordnung von mindestens 37–85 min und maximal 102–227 min angesetzt werden. Einen weiteren Beleg für die hier angenommene Reihenfolge der Chromatin-DNS-Synthese erhielt Ribas-Mundo (22) in der Beobachtung, daß die erste DNS-

Synthese, die in der Lymphozytenkultur nachzuweisen ist, die Nukleolen ausläßt.

Die unterschiedliche Dauer der G_2 Phase könnte erklären, warum bei der von uns gewählten H3 Thymidin- und Colcemid zeit von 6½ Stunden ein kleiner Teil (2%) der Zellkerne mit geringer Markierung (unter 100 Silberkörner) eine überwiegende Heterochromatinmarkierung aufweist (Abb. 3)

Der Entwurf eines Zeitplans der Reduplikation der Chromatintypen geht davon aus, daß die DNS-Synthese am Euchromatin beginnt und am Heterochromatin abschließt und daß dazwischen eine längere Phase liegt, in der sich Eu und Heterochromatinverdoppelung überschneiden. Dem Anteil der einzelnen Markierungstypen bei der Kurzzeitmarkierung (30 min) müßten demnach Zeitabschnitte innerhalb der S-Phase entsprechen (Abb 6) Nach diesen Befunden wird während der ersten 1,3 Stunden nur Euchromatin, während der *folgenden 7 3 Stunden Eu und Heterochromatin* und in den letzten 0 9 Stunden nur Heterochromatin synthetisiert. Diese Werte konnen nur als Schätzungen angesehen werden und sollen das Verhältnis der Zeitphasen zueinander demonstrieren.

Indem an heterochromatischen Anteilen des Interphasekerns die letzte DNS-Synthese vor Abschluß der S-Phase stattfindet, müssen diese Anteile den spätreduplizierenden Chromosomen und Chromosomenabschnitten entsprechen. Die Autoradiographie der Metaphasechromosomen der Leukozytenkultur nach Inkorporation von H3 Thymidin im letzten Teil der Synthesephase ergibt ein charakteristisches Muster (8, 24 7 5) Neben dem einen X-Chromosom der Frau sind bestimmte Autosomenabschnitte weitgehend



Abb. 6. Zeitplan der DNS Verdoppelung des Interphase-Chromatins. Die Zahlen geben die Prozentsätze an, in denen 3 verschiedene autoradiographische Markierungsmuster nach 30 min Inkorporation von H3 Thymidin zu sehen sind. Den Prozentsätzen entsprechen Zeitabschnitte innerhalb der S-Phase (9 6 Stunden) Demnach würde in den ersten 1,3 Stunden (14) vorwiegend Euchromatin synthetisiert, in den folgenden 7,3 Stunden 76) Eu- und Heterochromatin und in den letzten 0,9 Stunden (10) fast ausschließlich Heterochromatin

Euchromatin-DNS-Synthese
Heterochromatin-DNS-Synthese

konstant markiert. Die *Chromosomenautoradiographie ergänzt* inzwischen die Morphologie in der Identifikation von Extrachromosomen bei Trisomien und Translokationen und im Auffinden translozierter Stücke.

Die Mehrzahl der Chromosomenaberrationen beim Menschen betrifft Duplikationen und Defizienzen spätreduplizierender dem Heterochromatin entsprechender Chromosomenabschnitte. Die Zusammenhänge zwischen Chromatinmuster verzögerter Reduplikation, genetischer Aktivität und Zelldifferenzierung (26) enthalten noch viele offene Fragen

Zusammenfassung

Die DNS-Synthese im Interphasekern von Lymphocyten in der Kultur beginnt euchromatischen und endet heterochromatischen Strukturen. In der mittleren S-Phase findet sie an Eu- und Heterochromatin gleichzeitig statt. Diese Reihenfolge ergibt sich aus der toradiographischen Markierung nach Zugabe on H3 Thymidin und *Colcemid 6½ Stunden vor der Beendigung der Kultur Es wurde ein Zeitplan des Re*duplikationsablaufs entworfen. Außerdem konnte die Übereinstimmung spätreduplizierender Chromosomenabschnitte mit interphasischem Heterochromatin gezeigt werden.

Summary

Synthesis of DNA in the interphase ucleus of leukocytes in culture begins with euchromatic structures and ends with heterochromatic ones. I the middle S phase it takes place both in euchromatin and in heterochromatin t the same time This order has been shown by autoradiographic marking after Hx-labeled thymidine and colcemide *had* been added to the culture 6½ hours before terminating *the latter* A time table for the replication process was outlined. The late-replicating chromosomal segments were also shown to coincide with interphase heterochromatin.

Résumé

La synthèse de l'ADN dans le noy de l'interphase de lymphocytes de culture est liée à son début à des structures euchromatiques et à sa fin à des structures hétérochromatiques. Dans la phase S moyenne lle l'est en même temps à l'euchromatine et à l'hétérochromatine La suite chronologique de ces faits a été mise en évidence par marquage utoradiographique à l'aide de thymidine tritiée et l' djonction de colcemid 6½ heures vant la fin de la culture U horaire de la réduplication est esquissé En plus la concordance de segments de chromosomes se dédoublant tardivement ec l'hétérochromatine interphasique put être démontrée

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Fractionation of Erythropoietin by Selective Membrane Permeability*

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Erythropoietin exists in a free and bound form (1, 2). Less than 25 [illegible] of erythropoietin is normally free and diffusible (3). Most of the urinary erythropoietin seems to be bound to several proteins, and the binding limits the use of chromatography in the purification of the active principle. Erythropoietin has been demonstrated to be somewhat diffusible under certain conditions (4, 5, 6). Lewis *et al.* (3), working with urine concentrates obtained by flash evaporation, showed that small amounts of erythropoietin dialyzed through certain membranes. If the quantity of diffusible erythropoietin could be increased, the technique possibly would become a feasible approach to the purification of the active erythropoietin principle.

This report denotes a method for increased amounts of diffusible erythropoietin and examines the dialysis technique for feasibility as part of a fractionation procedure and a modification of the bioassay for erythropoietin.

Materials and Methods

Urine collected from patients with [illegible] severe erythroid bone marrow hypoplasia was chromatographed as described previously (7) and the fraction II [illegible] III obtained [illegible] dialyzed. The dialyzing [illegible] tubing* was 18/32, 20/32, 24/32, 1 [illegible] 1 [illegible] 1 [illegible] and 2 inch inflated diameter, regenerated or viking cellulose. The concentrated urine [illegible] which [illegible] one molar in NaCl [illegible] dialyzed with stirring at 2 C [illegible] against an equal [illegible] volume of distilled

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Supported in part by Grant No. AMO77[illegible] from the Institute of Arthritis and Metabolic Diseases, HE 10 91 and MO-1 FR-61-03, National Institutes of Health.

The tubing indicated by an asterisk was obtained from Arthur H. Thomas Co., Philadelphia, Pa., and the other tubing listed was obtained from Emil Greiner Co., New York, N. Y.

The material in this paper was presented [illegible] the meeting of the American Association for the Advancement of Science in Washington, DC., December [illegible] 19[illegible]



Fig 1 Desiccators simulating altitude (about 24,000 ft.) chambers. The gas mixture was 8 oxygen and 92 nitrogen.

ater for 4 days. Erythropoietin activity was determined on the urine concentrate, the non-diffusible portion and the diffusate after dialysis by modification of the bioassay for erythropoietin as described by PAVLOVIC KENTERA *et al.* (8)

Five desiccators, each housing 12 AKR 6 week old female mice, were joined in tandem and used to simulate altitude chambers. The mice were rotated to different positions three times week to assure an even consumption of oxygen. A tank of 8% oxygen and 92% nitrogen was connected to the desiccators by way of flow meter A flow rate of 2 ½ l/min and about 136 h of intermittent exposure to these hypoxic conditions, over period of 14 days, was used to produce polycythaemia sufficient for the erythropoietin assay Three and 4 days after the mice were removed from the simulated altitude chambers, they were injected intraperitoneally with 0.5 ml of the material to be tested or with control solutions such as saline or our laboratory erythropoietin control (7) Beyond this step the method was essentially the same as the one described by PAVLOVIC-KENTERA *et al.* (8) The units of erythropoietin activity were the same as those defined by GOLDWASSER and WHITE (9)

Results

The blood samples from 20 AKR mice collected one week after removal from the desiccators, had haematocrit values of 63 1±0 5 standard deviation of the mean) and haemoglobin concentrations of 19 0±0 4 g After injection of 1 ml of saline, 20 polycythaemic mice showed 0 06±0 00 units of erythropoietin activity per ml of blood After injection of 1 mg of our laboratory erythropoietin control 12 polycythaemic mice showed 1 01±0.06 units of erythropoietin activity per ml of whole blood therefore, the

Table I

Equilibrium dialysis of erythropoietin at one molar NaCl. Total activity

Saline control activity units	Urine concentrate activity units/ml	1 / tubing nondiffusible-diffusate activity units/ml		1 / tubing nondiffusible-diffusate activity units/ml	
0.08 ± 0.01	0.62 ± 0.07	0.51 ± 0.02	0.17 ± 0.01	0.47 ± 0.02	0.33 _ 0.01

Erythropoietin activity units as described previously with the standard deviation of the mean of 7 runs.

Table II

Equilibrium dialysis of erythropoietin at one molar NaCl. Total activity

Exp. No.	Membrane inches (I.D.)	Urine concentrate activity units /ml	Nondiffusible activity units/ml	Diffusate activity units ml
1 (7)	18/32	0.42 ± 0.02	0.36 ± 0.01	0.03 ± 0.02
2. (7)	20/32	0.39 ± 0.01	0.42 ± 0.04	0.01 ± 0.00
3. (7)	24/32	0.49 ± 0.02	0.44 ± 0.03	0.01 ± 0.01
4 (7)	1 /	0.50 ± 0.03	0.50 ± 0.06	0.05 ± 0.00
5. (9)	2	0.42 ± 0.03	0.26 ± 0.01	0.09 _ 0.01

Activity units as previously defined with the standard deviation of the mean.
Number of runs with 5 mice for each assay
I.D. = inside diameter

used method appears sensitive and reproducible. The normal haematocrit and haemoglobin concentration for 10 AKR mice was 45.2 ±1 4 % and 15 4 ± 0 3 g% respectively

Data from the assay of urine erythropoietin concentrates, the nondiffusible portions and diffusates during dialysis at high ionic strength are shown in Table I The erythropoietin activities of the diffusates were 2–4 times the saline control levels during equilibrium dialysis. After subtracting the saline control it can be seen that about 17 of the activity dialyzed through $1^1/_8$ inch tubing while about 46 of the activity dialyzed through $1^7/_8$ inch tubing The other membranes tested showed a relatively small amount of activity in the diffusates (Table II)

The data in Table I were calculated on the basis of units of activity per mg of nitrogen and re-examined. The specific activity of the erythropoietin in the diffusate from the $1^1/_8$ inch tubing was about the same as the non-diffusible portion and the urine concentrate whereas that from the $1^7/_8$ inch tubing was about twice any of the other activities

Discussion

The results showed the reliability of the modified bioassa erythropoietin in that the simulated altitude chambers produ polycythaemia by hypoxia as seen by the haematocrit and haer globin data.

The total activity of the non-diffusible portion and diffusate for the $1^7/_8$ inch membrane, increased to 133% of the initial activity (Table I) indicating the removal of an inhibitory substance as postulated by GARDNER *et al.* (10) The variable results among the dialysis data were due possibly to variations in dialysis membranes (11)

Some commercial cellulose membranes can be used to demonstrate selective permeability to erythropoietin at increased ionic strength. The bonds holding erythropoietin in the form of a complex seem to be ruptured by high salt concentrations sufficiently to allow a significant amount of erythropoietin to become diffusible through selected membranes. In view of such behavior it would appear that erythropoietin forms a complex with other proteins by means of hydrogen bonds, hydrophobic bonds, van der Waals forces and/or ionic bonds.

The dialysis of erythropoietin through the $1\,/_8$ inch membrane did show some advantage as part of a purification scheme, since the specific activity of the erythropoietin in the diffusate increased twofold some erythropoietin was escaping but some other proteins were being retained preferentially Since the erythropoietin specific activity of fraction II+III was increased about fi efold by chromatography (7) the twofold increase of specific activity in the diffusate represented an overall tenfold increase.

In a previous report (7) a 2 inch (inflated diameter) regenerated cellulose membrane was used to remove the salt from a urinary erythropoietin concentrate The 2 inch membrane has been discontinued by the distributor However an appropriate membrane can be selected from the data in Table II

Summary

A modified bioassay was used to study the permeability of dialyzing membranes to erythropoietin. Most of the membranes were only slightly permeable but one of the membranes showed selective permeability to erythropoietin with twofold increase in specific activity in the diffusate

senen Röhrchen wurden geschüttelt, in Eiswasser gekühlt und in der Kühlzentrifuge bei +4 °C 15 min bei 3000 rpm zentrifugiert. Die Plasmen wurden bis zur Bestimmung bei —30° gelagert. Gleichzeitig wurde Blut zur Bestimmung der Fettwerte abgenommen, wobei 1 Tropfen Heparinlösung/10 ml Blut vorgelegt war, um das Blut ungerinnbar zu machen.

2. Alle Bestimmungen wurden mit TRIS-Puffer pH 7,5, Ionenstärke 0,15, durchgeführt. Alle Lösungen wurden vor ihrer Verwendung mit einem Beckman-pH-Meter (Glaselektroden) eingestellt. Flüssige Reagentien wurden bei —30° gelagert.

3. Als Caseinlösung verwendeten wir Caseinum purissimum Merck nach Hammersten 6% (w/v). Die Lösung enthielt außerdem $7,5 \times 10^{-4}$ M Epsilonaminocapronsäure Fluka AG Buchs SG. Endkonzentration 3×10^{-4} M.

4. Streptokinase: Streptase Behringwerke, Fläschchen zu 250000 Christensen-Einheiten in 125 ml einprozentiger Caseinlösung (2000 E/ml) und in Portionen zu 10 ml eingefroren.

5. Rinderenglobulinpräzipitat (Plasminogenspender): Rinderplasma wurde 1:10 mit Aqua dest. verdünnt und bei pH 5,2 zweimal gefällt. Das abzentrifugierte Präzipitat wurde lyophilisiert und bei +4° C im Kühlschrank gelagert. Zum Versuch wurde eine zweiprozentige Lösung verwendet (ca. 5 RCE = Remmert-Cohen-Einheiten).

6. Alle Glaswaren wurden mit Desicote (Beckman) nach Angaben des Herstellers silikonisiert.

7. Trichloressigsäure purissimum p. A. Merck 10% (w/v) = TCA.

8. Abtrennung der Chylomikronen: 3 ml Plasma wurden in einer M.S.E. Superspeed 25000-Ultrazentrifuge bei 25000 bis 28000 rpm eine Stunde lang zentrifugiert, das chylomikronenfreie Plasma abgesaugt, die Chylomikronen zweimal in 0,9% Kochsalzlösung gewaschen und jeweils durch Zentrifugieren aufgerahmt sowie schließlich in 3 ml TRIS-Puffer suspendiert. Die Proaktivatorbestimmung im Plasma vor und nach dem Entrahmen sowie der aufgerahmten und in TRIS-Puffer suspendierten Chylomikronen erfolgte nach Punkt 9.

9. Proaktivatorbestimmung: Wir verwendeten eine von Alkjaersig et al. (1) angegebene Methodik mit einigen Modifikationen.

0,2 ml 1:40 mit TRIS-Puffer verdünntes humanes Test-Plasma (oder 0,1 ml Triglyceridsuspension) wurden in 1,8 ml Rinderenglobulinlösung gebracht und im Wasserbad auf 37 °C erwärmt. Nach Temperatureinstellung wurde 1 ml Streptaselösung (2000 E) dazugegeben. Die Mischung wurde genau 5 min (Stoppuhrkontrolle) inkubiert und dann 2 ml 6%ige Caseinlösung hinzupipettiert. 2 und 32 min nach Caseinzugabe wurden je 2 ml des Reaktionsgemisches entnommen und in 2 ml 10%iger TCA gefällt. Die Proben wurden nach mehrstündigem Stehenlassen bei Raumtemperatur 15 min bei 3000 rpm zentrifugiert, filtriert und das lösliche Tyrosin im Filtrat bei 280 mμ Wellenlänge in einem Beckman-D U-Spektralphotometer gemessen. Der Proaktivatorspiegel wurde als aktiviertes Rinderplasminogen in RCE* angegeben, wobei eine RCE als die Plasminmenge definiert ist, die in einer Minute in 1 ml 7,5 μg Tyrosin freisetzt. Es wurden Doppelbestimmungen durchgeführt und bei jeder Versuchsserie entsprechende Leerwerte als Kontrollen mitgeführt.

10. Zur Bestimmung der Blutfettwerte wurde das entnommene (Heparin-)Blut 10 min bei +4 °C und 3000 rpm zentrifugiert. Das abgehobene Plasma wurde nach Folch et al. (6) in der Modifikation von Carlson (4) extrahiert.

Im Extrakt wurden der Lipoidphosphor nach Bartlett (3) (Lipoidphosphor x 25 = Phospholipoide in mg%), das Gesamtcholesterin nach Searcy et al. (10), die Triglyceride nach Carlson (4) und die freien Fettsäuren nach Dole et al. (5) in der Modifikation von Trout et al. (13) bestimmt.

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* RCE = Remmert-Cohen-Einheit

Ergebnisse

1. Proaktivator-Spiegel bei Normalpersonen $N = 15$ $\bar{x} = 255{,}00$ RCE, $s = \pm 32{,}86$ RCE bei Hyperlipämikern (Tabelle I) $N = 15$ $\bar{x} = 309{,}26$ RCE, $s = \pm 48{,}98$ RCE. Die mittels Fischers F-Test durchgeführte Prüfung auf Streuungshomogenität betrug $F = 2{,}09$ $F = 2{,}48$ wodurch die Streuungshomogenität gesichert ist. Der Vergleich der Mittelwerte erfolgte hierauf mit dem T-Test nach STUDENT. Es wurde bei $F = 28$ für t der Wert 3,605 gefunden, der Unterschied ist daher mit einem $p < 0{,}0025$ gesichert.

2. Bei 8 Patienten mit einem besonders hohen Gehalt an Chylomikronen (Triglyceridwerte zwischen 1500 und 3000 mg%) wurde der Proaktivatorspiegel vor und nach Entrahmen durch die Ultrazentrifuge gemessen (Tabelle II). Er war im Mittel vor Entrahmen $\bar{x} = 293{,}87$ RCE, nach Entrahmen $\bar{x} = 313{,}37$ RCE, Differenz $= 19{,}50$ RCE $sD_1 = 20{,}90$ RCE. Die Differenz erscheint mit einem $p < 0{,}025$ statistisch nicht gesichert. Sie könnte aber aus dem Fehlen von 2–7 Vol% Chylomikronen erklärt werden.

3. Die Proaktivatorbestimmung der aufgerahmten und zweimal mit TRIS-gepufferter physiologischer Kochsalzlösung gewaschenen Chylomikronen ergab trotz zwanzigfacher Konzentration im Untersuchungssystem Werte, die sich von den Leer-Kontrollen nicht unterschieden.

4. Eine Korrelation zwischen dem Proaktivator-Spiegel und der Höhe einer der untersuchten Fettfraktionen (Triglyceride, Cholesterin, Freie Fettsäuren und Phospholipide) konnte nicht gefunden werden.

Diskussion

Unsere Untersuchungen ergaben bei den Patienten mit essentieller Hyperlipämie gegenüber randomisierten Normalpersonen (Unterscheidungskriterium Triglyceridspiegel von 500 mg% und darüber) eine statistisch gesicherte Erhöhung des Blut-Proaktivator-Spiegels im fibrinolytischen System. Es bestand aber weder bezüglich der Höhe der Triglyceride noch der übrigen Fettfraktionen (Cholesterin, Freie Fettsäuren, Phospholipide) eine direkte Korrelation.

Die Ansicht von GRECO *et al.* (7) die mit Hilfe der Fibrinplattenmethode über Proaktivatoreigenschaften der Chylomikronen

Tabelle I
Fettfraktionen und Proaktivator bei Hyperlipämikern (geordnet nach Triglyceridwerten).

Nr	Name	Chol.	Triglyz.	FFS	Ph-Lp.	RCE
1	Sen	1216	5900	1500	1190	240
2	Rie	311	2525	1100	795	351
3	May J	430	1960	763	640	322
4	Ede	350	1790	1180	570	273
5	Baa	665	1765	1707	635	352
6	Sch	600	870	540	494	315
7	Str	370	865	1250	443	330
8	Weg	590	765	710	483	263
9	Sta	570	720	715	412	255
10	May W	278	630	1280	475	333
11	Schm	431	610	630	411	385
12	*Rau*	336	*540*	*690*	*403*	*227*
13	Ebn	495	535	940	493	280
14	Ber	352	503	1100	398	340
15	Lug	432	495	745	491	360

Chol. Cholesterin, mg%
Triglyz. Triglyzeride, mg%
FFS Freie Fettsäuren, meq/l
Ph-Lp. Phospholipide, mg%
RCE Remmert-Cohen-Einheiten des aktivierten Rinderplasminogens (als Ausdruck des Proaktivators)

Tabelle II
Proaktivator Werte bei Hyperlipämikern vor und nach Entrahmen im Plasma, in Remmert-Cohen-Einheiten (RCE) des aktivierten Rinderplasminogens.

Nr	Name	Vor Entrahmen	Nach Entrahmen
1	Sen	240	261
2	Rie	351	390
3	May J	322	375
4	Ede	273	283
5	Sch	315	328
6	Str	330	315
7	Weg	263	290
8	Sta	255	265

berichteten, konnte in unserer Versuchsanordnung nicht bestätigt werden Der Proaktivator Spiegel bei unseren Patienten mit extrem hohem Chylomikronengehalt zeigte nach dem Entrahmen keine Verminderung auch gewaschene Chylomikronen in sehr hoher Konzentration ließen nach Streptokinase Aktivierung keine Pro-

aktivatoreigenschaften erkennen. Eine Vortäuschung fehlender Proaktivatorwirkung durch Inhibitoren scheidet weitgehend aus, da wir zeigen konnten, daß gewaschene Chylomikronen in einer Konzentration bis 6000 mg% keinen Antiplasmineffekt besitzen, der das System stören könnte.

Die Frage nach der pathophysiologischen Ursache des erhöhten Proaktivators bei den Hyperlipämikern läßt sich aus den bisherigen Ergebnissen nicht sicher beantworten. Es bestehen bekannt lich noch erhebliche Unklarheiten über die Natur des Blut Proaktivators (11) Der Proaktivator-Spiegel verhält sich zur endogenen Blutfibrinolyse indirekt proportional. Bei ihrer Steigerung durch Fieberschock oder Adrenalininfusion wies Müllertz (9) ein Absinken des Proaktivator-Spiegels innerhalb einiger Stunden bis auf 30°/ des Ausgangswertes nach. Es wäre demnach vorstellbar daß ein erhöhter Proaktivator-Spiegel mit einer Hemmung der Fibrinolyse in Zusammenhang steht. Als Erklärung könnte man eine Hemmung der Umwandlung vom Proaktivator zum Aktivator durch die pathologischen Fettfraktionen annehmen, die entweder die Freisetzung der Lysokinasen aus dem Gefäßwandendothel inhibieren oder direkt bei der Umwandlung Proaktivator–Aktivator interferieren (8*) Diese Hypothesen werden zur Zeit geprüft.

Zusammenfassung

Bei 15 essentiellen Hyperlipämikern (Triglyzeridwerte zwischen 495 und 5900 mg%) wurde der Spiegel des Blut Proaktivators im fibrinolytischen System an aktivierten Plasminogen durch Caseinolyse untersucht und ein signifikant erhöhter Proaktivator Spiegel gefunden.

Summary

The level of blood proactivator in the fibrinolytic system was investigated by caseinolysis on activated plasminogen in 15 patients with essential hyperlipaemia (triglyceride values between 495 and 5900 mg*/); significantly raised level of proactivator was found.

Résumé

Chez 15 malades atteints d'hyperlipémie essentielle (taux des triglycérides allant de 495 à 5800 mg*), le taux du proactivateur sanguin du système fibrinolytique été étudié à l'aide de la caséinolyse du plasminogène activé. Le taux de proactivateur était augmenté de façon significative.

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'Pitfalls of Factor VIII Assay*

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Factor VIII activity has been assayed by a variety of methods (1) and the results obtained seem to indicate that it is very difficult to define the normal plasma level

The purpose of our study was to analyze the results obtained by the thromboplastin generation test in normal individuals without reference to a standard to determine the reproducibility of the results to identify at least some of the variances which might influence the assay

Material

The investigation has been carried out in 50 normal individuals 25 males and 25 females of ages varying from 21 to 64 years. The samples have been obtained from doctors, medical students, nurses, tecnicians and aides of our department. I few cases the blood has been donated by patients affected by unrelated disorders.

Method

Reagents

(1) Platelet-poor citrated plasma of the subject under investigation was used as substrate-plasma.

(2) Platelin (Warner-Chilcott) was used as platelet substitute the material of one vial was dissolved in 2.5 ml of distilled water according to the directions given by the manufacturer

(3) Beef-serum prepared as described by Ware and Seegers (2) diluted 1 100 was used as the source of Factor V

The serum was distributed in 0.5 ml vials and frozen t −30 °C. The material, once thawed, was not refrozen. Some of the assay were performed using Difco-beef serum. Using either reagents the results were the same.

(4) Aged human oxalated serum was used as the source of serum factors (3) The serum obtained from the blood collected from normal donor without tissue thromboplastin, was distributed in 1 ml vials and frozen t −30 °C. After thawing the material was not refrozen. The serum was diluted 1 10 and kept t room temperature for 1 h before testing

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* Preliminary results of this work have been presented at the meeting of the 'Società Italiana di Ematologia on December 4th, 1965 in Milano.

(5) $Al(OH)_3$ adsorbed plasma was used as the material to be assayed for factor VIII activity. The adsorbed plasma was diluted from 1:5 to 1:320.

(6) Beef serum, aged human serum and $Al(OH)_3$-plasma were diluted in 0.85% NaCl solution.

(7) Sodium citrate 3.8% was used as anticoagulant (1 vol. + 9 vol. of blood).

(8) $CaCl_2$ M/40 solution.

Procedure

(1) Blood was collected using two syringes.

(2) The platelet-poor-plasma, obtained by centrifuging, was divided in two aliquots. One was used as the substrate, the second one was adsorbed with $Al(OH)_3$ suspension (2.5%); both aliquots were kept at +4°C during the time of the assay.

(3) The incubation mixture was prepared by adding to a tube kept at 37°C 0.4 ml of each of the following reagents: platelin suspension, aged human serum, beef serum, $Al(OH)_3$ plasma diluted from 1:5 to 1:320 or 0.85% NaCl solution as it will be indicated in the description of the different steps of the assay.

(4) 0.1 ml of substrate plasma were distributed in tubes kept at +37°C.

(5) The incubation mixture was recalcified adding 0.4 ml of $CaCl_2$.

(6) At the intervals indicated in the description of the various steps of the assay 0.1 ml of the incubation mixture and 0.1 ml of $CaCl_2$ were transferred into the tubes containing the substrate plasma and the clotting time recorded. In preliminary experiments we have tested the clotting time of the substrate plasma using either beef serum or $Al(OH)_3$-haemophilic plasma as a source of factor V in the incubation mixture. The results seem to indicate that the beef serum would be a more suitable reagent: in fact a shorter substrate-plasma clotting time is obtained by using the beef serum (18 and 31 sec respectively).

The factor VIII assay has been carried out in 3 parts.

In the first set of determinations the clotting activity of the incubation mixture substituting the $Al(OH)_3$-plasma with 0.85% NaCl solution has been determined. After recalcification, 0.1 ml of the incubation mixture and 0.1 ml of $CaCl_2$ were transferred, at intervals of 3 min for 30 min, into the tubes containing 0.1 ml of the substrate-plasma and the clotting time recorded. Here we have determined the clotting time of the substrate-plasma which should correspond to a level of factor VIII activity equal to zero. This experiment has been performed in 32 of the 50 individuals examined. The substrate-plasma clotting time varied between 16 and 124 sec with an average value of 57 sec ($\delta = 20$; $\delta/\mu = 0.35$).

Then the optimal incubation time of the incubation mixture has been determined using the $Al(OH)_3$-plasma to be assayed diluted 1:5. The incubation time, corresponding to the shortest substrate plasma clotting time, has been selected for the assay.

Thirdly the various dilutions (from 1:5 to 1:320) of the plasma to be assayed were tested. The incubation mixtures were prepared in 7 tubes, kept at +37°C, using the various dilutions of the $Al(OH)_3$-plasma. At the optimal incubation time 0.1 ml of the incubation mixture and 0.1 ml of $CaCl_2$ were transferred into the tubes containing the substrate plasma and the clotting time recorded. The incubation time, previously selected, was constant for each dilution.

Results and Discussion

The results of this study summarized in Table I, indicate that the assay of factor VIII activity is subjected to such a variance

Table I

ng time of the substrate-plasma in seconds Al(OH)$_3$-plasma diluted as indicated in the incubation

Al(OH)$_3$-plasma substituted by 0.85% NaCl	Substrate plasma clotting time at the optimal incubation time	Al(OH)$_3$-plasma 1 5	1 10	1:20	1 40	1 80	1 160
16–124	8–52	9–90	11 116	14–106	17 120	30–129	43–131
57	27	37	47	57	69	79	82
20	15	18	23	22	—	—	—
0.35	0.56	0.49	0.49	0.38	—	—	—

ge value μ, standard deviation δ, variance coefficient δ/μ.

that it should be interpreted with great caution the average values (μ) and the standard deviations (δ) seem to indicate that the quantitative assay is not quite reliable. In fact the variance coefficient (δ/μ) is never below 0.35

The clotting activity of the incubation mixture in absence of Al(OH)$_3$ plasma seems to be related to the substrate plasma. In fact the clotting time of the substrate plasma of different individuals varies quite widely (μ = 57 sec, δ = 20 δ/μ = 0.35) Platelin suspension, aged human serum and beef serum used throughout this study belong to the same batch, therefore they should have a constant activity The clotting time of the substrate-plasma, obtained with various dilutions of the plasma to be assayed, should obviously fall within this limit with longer clotting time factor VIII activity should be considered zero.

In most of the cases only the first two or three dilutions give clotting time which might be considered valid. The test seems to fail to give reliable indications at levels less than 30% which is the most important area from the clinical standpoint. In fact the clotting times obtained with the Al(OH)$_3$-plasma diluted 1 20 give an average value (μ) = 57 sec.

Another important point is that the reproducibility is poor The optimal incubation time has been determined with Al(OH)$_3$ plasma diluted 1 5 in the incubation mixture. At the time of the assay the same dilution of the plasma has been retested During the time of the assay the plasma was kept at melting ice temperature nevertheless the substrate-plasma clotting time varied quite sensibly in the two determinations (μ = 27 and 37 sec respectively) The

poor reproducibility has been noted since the beginning of the experiments.

The results obtained from different individuals indicate quite strikingly the difficulty of choosing average values for each dilution. Some normal subjects, compared to others, should have no detectable factor VIII activity according to the results obtained.

The wide variance of the clotting time of the substrate-plasma obtained when *Al(OH)$_3$* plasma was substituted with *0.85%* NaCl solution and the poor reproducibility when the Al(OH)$_3$-plasma diluted 1:5 was retested, cannot be easily explained. The assay of factor VIII activity at least with this method seems to raise many questions.

Acknowledgements. We wish to thank Prof. C. F. Mamara from the Istituto Matematico 'Federico Enriques' of the University of Milan for his valuable suggestions.

Summary

Factor VIII activity has been assayed in 50 normal individuals by [illegible] modification of the thromboplastin generation test. The clotting activity of the incubation mixture varies with the individual substrate-plasma. Reproducibility with the same dilution of the Al(OH)$_3$-plasma is poor in spite of strictly controlled experimental conditions. Same dilutions of different plasma display such variance that a normal standard is virtually impossible. Results of factor VIII assay with this method should be interpreted with much caution.

Zusammenfassung

Bei 50 Gesunden wurde die Aktivität von Faktor VIII mit einem modifizierten Thromboplastingenerationstest bestimmt. Die Gerinnungsaktivität des Inkubationsgemisches variiert mit dem individuellen Substratplasma. Mit derselben Verdünnung des Al (OH)$_3$-Plasmas ist die Reproduzierbarkeit schlecht, trotz genau eingehaltener experimenteller Bedingungen. Dieselben Verdünnungen verschiedener Plasmen ergeben so stark wechselnde Resultate, dass es nicht möglich ist, einen normalen Standard aufzustellen. Die Resultate der Faktor VIII Bestimmung mit dieser Methode sollten mit grosser Vorsicht interpretiert werden.

Résumé

L'activité du facteur VIII a été déterminée chez 50 personnes normales à l'aide d'une modification du test de formation de la thromboplastine. L'activité de coagulation du mélange incubé varie selon le plasma individuel servant de substrat. La reproductibilité des résultats obtenus avec la dilution de plasma Al(OH)$_3$ est faible malgré des conditions expérimentales rigoureusement contrôlées. Quelques-unes des dilutions des différents plasmas employés présentèrent une telle variabilité qu'il est virtuellement impossible d'établir un standart normal. Les résultats de la détermination du facteur VIII obtenus à l'aide de cette méthode doivent être interprétés avec beaucoup de prudence.

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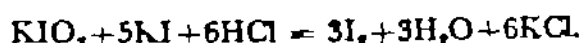
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Electron Microscopic Examination of Hemagglutination Produced by Iodinized Coombs Serum

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Our investigations were based on the notion that the electron density of iodinized proteins increases because of the high atom number of the iodine ion added. Accordingly iodinized immune globulins may become visible in the electron microscope. On iodinization of proteins the iodine will be bound to the tyrosine ring (4). Francis *et al.* (3) used the following basic formula for the iodinization of proteins



$$KIO_3 + 5KI + 6HCl = 3I_2 + 3H_2O + 6KCl.$$

The liberated iodine does not damage the proteins, whereas hydrochloric acid can be neutralized with phosphate buffer. Bourinell *et al.* (2) labelled antisera with radioactive I^{131}. In our studies, we used this method with the only difference that instead of radioactive iodine salts we used normal iodine in the reaction. According to Baxandall (1) iodine is (besides ferritine, mercury and uranium) one of the substances suitable for the visualisation of antibodies.

Methods

The solutions used in our experiment are: (1) Potassium iodate: 0.107 g KIO_3 p. a. in 100 ml bidestilled water. (2) Potassium iodide solution: 0.415 g KI p. a. in 100 ml bidestilled water. (3) 0.1 N HCl. (4) Na_2HPO_4-buffer solution (0.2 mol): 3.56 g $Na_2HPO_4 \cdot 7H_2O$ p. a. solved in 80 ml bidestilled water, brought to pH 8.0 with p. HCl and adjusted to 100 ml final volume.

Iodinization of the Coombs serum and of the control serum was made as follows: 0.11 ml KIO_3 (0.107 g/100 ml) and 0.1 ml KI (0.415 g/100 ml) solution were placed in a test tube and then 4 drops of 0.1 N HCl were added. Owing to the effect of the HCl, iodine was liberated from the potassium iodate-potassium iodide system which stained the solution brown. The pH of the solution was increased by adding 2 drops of

0.2 M phosphate buffer (pH 8.2) then 0.5–2.0 ml of antihuman globulin rabbit serum was added very quickly Then it was left to stand for 90 min which was to ensure the completeness of iodination. After this the free iodine in the surplus was bound with few drops of 0.1 M sodium thiosulphate and the solution so prepared was then dialyzed at 1–4 °C against 500 ml physiological NaCl. Dialysis was repeated five times with fresh physiological NaCl solutions. After this preparation the serum was used for serological reactions, in this case for Coombs test with sensitized human red blood cells. Iodinization of normal serum as control was performed using the same method. Rh-positive red blood cells were then sensitized with anti-Rh (anti-D) serum. The red blood cells so treated were washed with physiological saline three times and put in test tube together with non-iodinized Coombs serum as well as with iodinized normal serum according to Coombs test.

The agglutinate of the red blood cells treated with specific iodine-labelled serum, the control red blood cells treated with iodinized normal serum and the conglomerates of the red blood cells treated with non-iodinized Coombs serum were fixed in Millonig buffered osmium tetroxide for an hour rinsed in the buffer solution, dehydrated in graded series of alcohols and embedded in araldite. Ultrathin sections were made on LKB and Tesla ultramicrotomes and examined with Tesla 242 D table electron microscope

Results

On the surface membranes of the red blood cells treated with the specific and iodine labelled sera (Coombs serum) there were several (4–8) 500–1000 Å electron dense plaques to be seen (Fig 1) Such plaques could be seen not at all in the control red blood cells treated with iodine labelled normal serum and they could never be observed on red cells treated by the non-iodinized Coombs-serum.



Fig 1

These findings prove directly that the binding of the antigen to the antibody in this case the binding between the anti-Rh-globuline and the antihuman immune globuline (rabbit immune globuline) occurred on the surfaces of the red blood cells.

Summary

Red blood cells sensitized with anti-D globulin were agglutinated with antihuman globulin rabbit serum labelled with normal iodine. By lectron microscopy the binding of the iodine-labelled immune globulins to the sensitized blood red cells was indicated by 500–1000 Å lectron dense plaques on the surface membranes.

Zusammenfassung

Mit Anti-D-Globulin sensibilisierte Erythrozyten wurden mit jodmarkiertem Anti-Humanglobulin-Kaninchenserum agglutiniert. Mit dem Elektronenmikroskop konnte die Bindung des jodmarkierten Immunglobulins an den sensibilisierten Erythrozyten in Form von 500–1000 Å messenden Belägen uf der Membran nachgewiesen werden.

Résumé

Des érythrocytes sensibilisés à l'aide d'anti-D-globulines ont été agglutinés avec du sérum marqué au jode normal provenant de lapins traités avec des globulines humaines. Au microscope électronique la liaison entre les immun-globulines marquées au jode et les érythrocytes sensibilisés est pparue sous forme de plaques imperméables aux électrons, qui se trouvent à la surface des globules et mesurent 500 à 1000 Å.

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Acta haemat. 38, 391-396 (1967)

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Two Ph1 Chromosomes in Blastic Crisis of a Granulocytic Leukaemia

M. H. Kilan and H. Martin

The fairly constant cytogenetic pattern of chronic granulocytic leukaemia (CGL) with its model number of 46 chromosomes and with its characteristic minute chromosome, known as Philadelphia (Ph1) chromosome (11), does not always prevail in the terminal phase of this disease. In the blastic crisis of CGL and in acute myeloblastic leukaemia (AML) there is no single or consistent chromosome aberrancy.

Recently some cases of CGL in acute crisis (3, 4, 5, 6, 7, 9, 13, 14), two cases in chronic phase (2, 13) and one case of AML (9) have been described in which two Ph1 chromosomes were present. Two of these cases showed double Ph1 chromosomes in diploid cells also, another two cases contained Ph1 disomy in hypodiploid cells as well, all other cases had two Ph1 chromosomes in hyperdiploid cells only.

We describe here a case of CGL in terminal phase with two Ph1 chromosomes in diploid and in hypodiploid cells. Another interesting finding in our patient was the presence of a ring chromosome in some metaphases. To the best of our knowledge this combination has not been described until now.

Case Report

A 29 years female was diagnosed as having CGL in 1964. She was treated in another hospital with busulfan and prednisone having 4 mg each per day on admission. Because of ineffective treatment she was referred to our clinic on 23rd March 1966. Clinical examination revealed "cushingoid" facies and numerous petechiae. The spleen was palpable 3 cm and the liver was enlarged 11 cm under the costal margin. Hb 8.5 g%, red cells 2.7 Mill/mm³, platelets 2700/mm³, white cells 27,200/mm³ with myeloblasts 92%. The bone marrow (23rd March 1966) was hypercellular with over 90% myeloblasts, 2 erythroblasts per 100 white marrow cells. The therapy was changed on admission. Prednisone 200 mg per day and 6-mercaptopurine 150 mg per day were given.

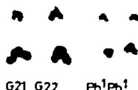


Fig. 1. Double Ph1 chromosomes from two bone marrow metaphases each containing 44 chromosomes. G 21 in upper row is abnormal showing deletion of the short arm.

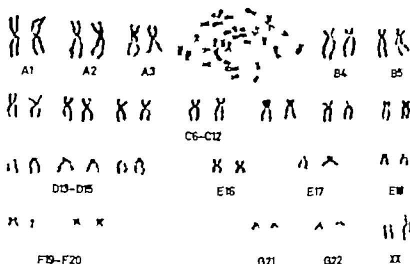


Fig. 2. Peripheral blood culture metaphase plate containing 46 chromosomes. Karyogram of the same metaphase showing two Ph1 chromosomes, one is arbitrarily located in G21 and the other in G22.

Because of very quick response of total white cells (800/mm³ on 26th March 1966) the doses were progressively decreased (prednisone 50 mg per day, 6-mercaptopurine was omitted. She died on 5th April 1966.

Cytogenetic Analysis

The cytogenetic analysis were performed on bone marrow using the direct air dry technique without prior in vitro culture (8) and on preparation from peripheral blood culture using a modification of the method described by MOORHEAD *et al.* (10). The blood culture was incubated for 51 h using Bacto-phytohemagglutinin (PHA). Colcemid (Ciba) was added during the final two hours to accumulate the mitoses. The bone marrow preparations were carried on 23rd March 1966 and the peripheral blood culture was incubated on 24th March 1966. The combined DENVER (1) and PATAU (12) system of nomenclature was used. 20 cells were karyotyped (Table I).

Table I
Karyotype analyses.

Cells karyotyped		Chromosome No.	Affected series, Paris nomenclature A	B	C	D	E	F	G	Other major findings
1	1	47				−1	+1	+1		Two Ph1 chromosomes
6	3	46								Two Ph1 chromosomes
	3	46								Ph1 chromosome negativ
4	2	45			−1					Two Ph1 chromosomes
	1	45					−1			Two Ph1 chromosomes
	1	45			−2					A ring chromosome
7	2	44			−2					Two Ph1 chromosomes
	1	44			−1		−1			Two Ph1 chromosomes
	1	44			−2	−1				Two Ph1 chromosomes + ring bromosome + fragment
	1	44			−1	−1				Ph1 chromosome negative
	1	44			−1		−1			Ph1 chromosome negative
	1	44			−4	−1			+2	A ring chromosome
1	1	43			−4	−1			+3	A ring chromosome
1	1	40			−4		−2			1 Ph1 chromosome + 1 fragment deletion of E16
Total no cells karyotyped 20	20									

One karyogram taken from bone marrow examination.
N. B. T polyploid cells had t least four Ph1 chromosomes.

Fig 3 Sections from eight different cells, section b is from bone marrow the others are from peripheral blood culture. ( ) Triple associations between DDG members and between D-series, adhesion of D with G member (*b*) Fusion and adhesion of chromosomes. ( ) Vacuole formation due to split of double spiral structure (major and minor spiral) of chromatid (*d*) Secondary constrictions. ( ) Spiralisation in early metaphase. (*f g*) Spiralisation in metaphase, (*g*) spiralisation is very irregular (*h*) Due to the absence of matrix or kalymma the intact frame work of the chromosome known as chromonema, is prominent. The chromonema contains densly stained bodies of arious sizes, known as chromomers. They are arranged in pairs and are connected by an understood fibre being capable of extension and liable to breakage.



Fig 4 V rious ypes of ring chromosomes from different metaphase plates of the same patient, first from the left is in prophase

Cytogenetic Results and Comments

Eight out of ten available bone marrow metaphases showed two Ph1 chromosomes (Fig 1) They were hypodiploid

Sixty metaphases from peripheral blood culture were microphotographed and analysed, 27% showed two Ph1 chromosomes (Fig 2) 10% of the diploid cells had two Ph1 chromosomes. Ph1 positive as well as Ph1 negative cells showed various structural abnormalities, e.g chromatid gaps, chromatid breaks, dicentric chromosomes, fusions of chromosomes (Fig 3b) vacuole formation in chromatid (Fig 3c) imperfect coiling (Fig 3e, f, g) and loss of matrix (Fig 3h) Chromosomal satellite associations (Fig 3a) were frequently observed The Ph1 negative diploid cells were most likely of lymphoblastic origin derived from lymphocytes through stimulation with PHA.

A predominant pathological cell line was considered with model number of 44 chromosomes. 37% of these cells in peripheral blood culture showed two Ph1 chromosomes.

18% of the hypodiploid cells presented ring chromosomes of variable size and shape. Karyograms having ring chromosomes (Fig 4) were bizarre

The presence of Ph1 disomy and their combination with ring chromosome may mean further progression of neoplastic disease. Their real significance is obscure.

Summary

A case of chronic granulocytic leukaemia in blastic crisis with two Ph1 chromosomes and ring chromosome is reported.

Zusammenfassung

In der terminalen Blastenkrise einer chronischen myeloischen Leukämie wurden 2 Ph-Chromosomen und ein Ring-Chromosom beobachtet.

Résumé

U cas de leucémie chronique myéloïde se trouvant dans sa crise de blastes terminale et présentant 2 chromosomes Ph et un chromosome en forme d'anneau est rapporté

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Chromosomal Abnormalities of Spleen Cells in Osteomyelosclerosis

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A few studies on chromosomal changes in osteomyelosclerosis have been published (2, 5, 8, 9, 10). They were performed using the method of karyotype analysis of bone marrow cells or cells from cultured peripheral blood. These investigations showed the presence of hyperdiploidy with group C trisomy (5, 10), hypodiploidy due to a random loss of chromosomes (5, 8) and their structural changes (2, 5).

The fibrotic and aplastic bone marrow on the one hand and leucoerythroblastic peripheral blood on the other hand do not represent the exact nature of osteomyelosclerosis, in which myeloid metaplasia of the spleen is the main feature.

No data have been presented so far concerning the chromosomal behaviour of spleen cells in myeloid metaplasia. The authors have undertaken the preliminary cytogenetic investigations of cells from cultured spleen aspirate and cells from cultured peripheral blood leucocytes in a case of osteomyelosclerosis.

Methods

Chromosome preparations were made from cells of cultured spleen aspirate and from cultured peripheral blood leucocytes according to the micromethod of Turpin and Lejeune (12) with slight modifications. The spleen material was obtained by aspiration in local anaesthesia. Spleen aspirate (0.2 ml) was immediately put into a sterile tube containing 10 ml of culture medium with 0.1 ml of Phytohemagglutinin M (Difco) and 0.05 ml of heparin.

Case Report

D. Z., a 53 year old female (N 944/313), was diagnosed as having osteomyelosclerosis in 1934 when she developped hepatosplenomegaly, leucoerythroblastic anaemia with generalized marrow aplasia and sclerotic changes of pelvic bones. Since than she

has been treated 5 times in the hospital because of severe anaemia and marked splenomegaly In the mean time she has been followed up in the Outpatient Department of the Institute of Haematology Since 1958 the patient was treated with intermittent courses of corticosteroids.

She was admitted to the hospital on May 21 1966 because of severe anaemia and generalized skeletal pains. Physical examination detected pallor and marked hepatosplenomegaly (the liver was palpable 10 cm below the right costal margin and spleen was palpable 15 cm below left costal margin)

At the time of chromosome study laboratory examination revealed haemoglobin 8.5 g/100 ml, RBC 3 300 000 mm³ WBC 78 000 mm with promyelocytes 11%, myelocytes 16 metamyelocytes 21 bands 20%, segmented 31 and lymphocytes 1 %. In peripheral blood there were found two erythroblasts per 100 leucocytes. Repeated attempts to obtain bone marrow were not successful. X ray picture of bones showed typical, generalized osteosclerosis. At the time of cytogenetic studies the patient was treated with blood transfusions and corticosteroids. Therapy with Busulfan was started after chromosome studies because of marked splenomegaly and increased white cell number

Results

The results of chromosomal analysis are shown in Table I It is apparent that the spleen cell culture reveals the chromosome abnormalities far more frequently than peripheral blood cells culture. The modal number of 46 chromosomes was observed in 76,9% of metaphases examined from peripheral blood cells and only in 29 4% of metaphases examined from spleen aspirate culture. More than 70% of spleen cell metaphases contained the aneuploid number of chromosomes in the form of hypodiploidy (Fig 1) and hyperdiploidy (Fig 2) In the latter the aneuploid metaphases were present in significant numbers. Hypodiploidy was due to a random loss of chromosomes. The hyperdiploidy in cells was dependent on the presence of extra chromosomes in group C and/or D One cell from peripheral blood culture and 2 cells from the spleen aspirate culture showed endoreduplication. The chromosomes of 2 cells from peripheral blood culture and 9 cells from spleen

Table I

Chromosome constitution of peripheral blood and spleen cells in case of osteomyelosclerosis.

Material	Chromosome number							Endoreduplication	Total count
	44	44	45	46	47	48	>48		
Peripheral blood cell culture	3	—	—	20	2	—	—	1	26
Spleen cell culture	3	—	5	10	7	3	2	2	34

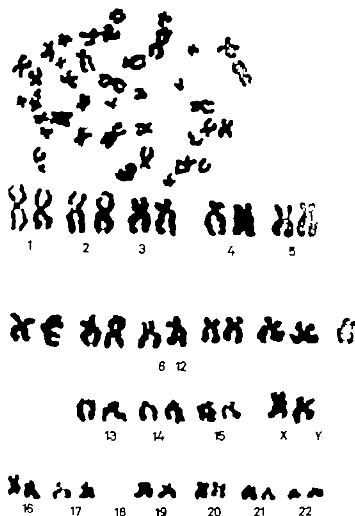


Fig 1 Metaphase and karyotype of cultured spleen cell containing 41 chromosomes.

aspirate culture showed precocious splitting at the centromeres (they are not included in Table I)

Discussion

In a patient with the typical history of osteomyelosclerosis of 12 years duration the cytogenetic investigations were carried out employing chromosome preparation made from cultured spleen



Fig 2 Metaphase and karyotype of cultured spleen cell containing 47 chromosomes.

aspirate The aneuploid number of chromosomes was found in more than 70 ⁄ of metaphases examinated. Hyperdiploidy was mainly due to an extra chromosome in the group C or/and D hypodiploidy was due to a random loss of chromosomes

It should be pointed out that at the time of the cytogenetic investigations, the patient was not in leukaemia like terminal phase of the disease and showed no blastic crisis of the disease. Likewise

she had received no previous treatment with radiomimetic drugs or ionizing radiation which might cause the karyotypic changes. She has not shown congenital or generalized chromosomal defect as indicated by the presence of normal diploid cells in the peripheral blood culture. No Ph chromosomes were found which are characteristic for the chronic myeloid leukaemia (1 7) The Ph chromosome has been found in almost all cases of chronic myeloid leukaemia, observed during all phases of this disease and has been unaffected by treatment (2, 11) Chromosomal abnormalities of the spleen cells observed by us might indicate that myeloid metaplasia of spleen in osteomyelosclerosis in spite of its rather mild course is more similar to neoplastic disease (4 6) and acute leukaemia (3) than to chronic myeloid leukaemia.

Concluding these results it is suggested that the chromosomal changes of proliferative cells in different neoplastic diseases, with various degrees of malignancy changing clinical course and cytological features, may be very similar

As far as we know this is the first report in the literature of supravital chromosome studies of the spleen cells in general and in osteomyelosclerosis in particular On the basis of these results it is emphasized that the examination of metaphases of spleen cells offers more suitable and reliable means of establishing chromosomal anomalies in osteomyelosclerosis than those permitted by the blood culture method since the spleen is the main site of myeloid metaplasia. Further cytogenetic investigations of the spleen cells in osteomyelosclerosis and comparison of results with those obtained in other proliferative disorders should widen our knowledge regarding chromosomal abnormalities and pathogenesis of these diseases

Summary

Chromosome investigations were carried out in a typical case of osteomyelosclerosis by the method of karyotype analysis of the cells from cultured spleen explants and from the peripheral blood leukocytes. There was sharp preponderance of diploid metaphases in cultured blood in contrary to the aneuploidy characterizing spleen explants culture. No Ph chromosomes were found. It is suggested that myeloid metaplasia of the spleen in osteomyelosclerosis is more similar with regard to chromosomal abnormalities to neoplastic disease and acute leukaemia, than to the chronic myeloid leukaemia.

Zusammenfassung

Bei einem Fall von Osteomyelosklerose wurden zytogenetische Untersuchungen vorgenommen mit Hilfe der Karyotyp-Analyse von Zellen aus einer Milzpunktatkultur

und von Leukozyten des peripheren Blutes. Es fand sich ein deutliches Überwiegen diploider Metaphasen in der Blutkultur im Gegensatz zu der für die Milzpunktatkultur charakteristischen Aneuploidie. Ph¹-Chromosomen waren nicht nachzuweisen. Es wird vermutet, daß die myeloische Metaplasie der Milz bei Osteomyelosklerose mit Bezug auf die Chromosomenanomalien den Neoplasmen und der akuten Leukämie näher steht als der chronischen myeloischen Leukämie.

Résumé

Dans un cas de ostéomyélosclérose, l'on a procédé à des examens cytogénétiques à l'aide de l'analyse du caryotype de cellules provenant de cultures de ponctions de rate et de leucocytes du sang périphérique. Dans ces dernières, il y avait une prépondérance des métaphases diploïdes, l'aneuploïdie caractérisant par contre les cultures obtenues par ponction de la rate. Aucun chromosome Ph¹ ne fut observé. L'attention est attirée sur le fait que dans l'ostéomyélosclérose la métaplasie myéloïde de la rate ressemble quant aux anormalités chromosomiques davantage à une maladie néoplasique et à une leucémie aiguë qu'à une leucémie myéloïde chronique.

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Libri

A. Ducla Soares – Francisco Parreira: Propedêutica Médica. I Volume/Hematologia. Fundação Calouste Gulbenkian, Lisboa. 500 pp.

Das vor allem für das portugiesische Sprachgebiet geschriebene Lehrbuch gibt eine gute Einführung in die klinische Hämatologie. Es besteht aus 7 Teilen über normale Blutbestandteile, Anämien, Polyglobulien, nicht leukämische Leukozytenveränderungen, Neoplasien, Milz und Knochenmark und hämorrhagische Diathesen. Die Kapitel und Unterabschnitte sind übersichtlich geordnet und behandeln den Stoff vollständig und doch in einer einfachen und für den Studenten leicht verständlichen Form. Auch seltene Anomalien und Syndrome sind berücksichtigt, und wenn biochemische Grundlagen aus verständlichen Gründen nicht allzu ausführlich abgehandelt werden, sind doch überall moderne Kenntnisse über die Pathogenese in den Text eingebaut. Der Band enthält 192 zum Teil farbige Abbildungen, ein kurzes Literaturverzeichnis über einschlägige Monographien und am Schluß ein übersichtlich gegliedertes Inhaltsverzeichnis, leider aber kein Sachwortregister. Für den Anfänger, der das Buch zur Hand nimmt, sind Kenntnisse der portugiesischen Sprache wohl unerläßlich. Wer aber in der Hämatologie etwas Bescheid weiß, versteht den Text auch mit italienischen oder spanischen Sprachkenntnissen recht gut.

H. R. Marti, Basel

J. M. Yoffey: Bone Marrow Reactions. Edward Arnold, London 1966. 152 pp. Preis 30 s.

In dieser Broschüre bezweckt der Autor die heutigen quantitativen und dynamischen Kenntnisse über die wichtigsten Knochenmarkzellen in Kürze darzustellen. Die einzelnen Kapitel betreffen die klinische und experimentelle Beeinflussung der Erythropoese durch Hypoxie, die Dynamik der Granulocyten unter normalen und pathologischen Verhältnissen, die eosinophilen Reaktionen, die basophilen Granulocyten und die Lymphocyten. Die Darstellung ist übersichtlich, der Text knapp und klar und nahezu frei von Spekulationen. Dem heutigen Zeitalter der quantitativen hämatologischen Dynamik liefert die Broschüre einen wesentlichen Beitrag.

P. Frick, Zürich

I. Boll: Granulocytopoese unter physiologischen und pathologischen Bedingungen. Experimentelle Medizin, Pathologie und Klinik, Band 17. Verlag: Springer, Berlin/Heidelberg/New York 1966. 185 S., VIII, 54 Abb., Preis DM 48.

Privatdozentin Dr. Irene Boll vom Städtischen Krankenhaus Berlin-Neukölln veröffentlicht hier ihre 1963 mit dem Frerichs-Preis ausgezeichnete Arbeit. Es handelt sich um eine gute Zusammenstellung der Ergebnisse mikroskopisch-morphologischer Forschungen über die Entstehung der neutrophilen Granulocyten mit vielen eigenen Beiträgen der Autorin. Die Monographie gliedert sich in 3 wichtige Teile. Der ausführlichste Teil ist der normalen Granulocytopoese gewidmet. Aus mathematischer Auswertung morphologischer Befunde und Autoradiographie-Versuchen wird eine neue Theorie über die Proliferation der Granulocytopoese entwickelt, die zwei gleichzeitig vorkommende Vermehrungsarten der unreifen Zellen annimmt. Dann folgen Kapitel über die pathologische Granulocytopoese bei chronischer Myelose, akuter Leukose und Perniciosa. Der kurze letzte Teil behandelt die Bedeutung der Granulocyten-Proliferation

für die Tumorbehandlung mit Cytostatica und spricht auf Grund der zellkinetischen Verhältnisse einer alternierenden zytostatischen Therapie das Wort.

Die Monographie ist übersichtlich gegliedert und enthält zahlreiche ausgezeichnete Mikrophotographien und am Schluß ein umfangreiches Literaturverzeichnis und ein nützliches Schlagwortregister. Sie kann jedem Hämatologen, der an Problemen der Granulozytopoese interessiert ist, empfohlen werden. H. R. Marti, Basel

Ch. G. de Boroviczény: *Standardization in Haematology III.* 3rd Transaction of the Int. Committee for Standardization in Haematology. Strasbourg 22nd and 27th August 1965. Bibl. Haem. Fasc. 24, VIII + 212 S., 33 Tab., 65 Abb. Preis sFr 50.—

Der dritte Band in der Folge der von Ch. G. von Boroviczény herausgegebenen Tagungsberichte der Internationalen Standardisierungskommission für Hämatologie liegt jetzt vor. Während die 1964 und 1965 erschienenen ersten zwei Bände vorwiegend Fragen der Hämoglobinbestimmung und allgemeine methodische Probleme behandelten, befaßt sich der neue Band größtenteils mit Blutkörperchenzählmethoden und Hämatokrit. Er enthält 18 Referate berufener Fachleute aus ganz Europa und den USA. Von den aktuellen Themata, die jeden Laboratoriumsleiter angehen, seien nur einige erwähnt: Temperaturbedingte Fehlerquellen bei der Zellzählung mit dem Coulter Counter, Volumenverteilungskurven roter Blutkörperchen mit elektronischen Zählgeräten, Thrombozytenzählung mit elektronischen Geräten, Herstellung von Standardsuspension zur Kontrolle der Zählapparate, Vergleich verschiedener Methoden zur Hämatokritbestimmung, Vorteile systematischer Hämatokritbestimmungen in der Klinik, direkte Zählung fetaler Erythrozyten im mütterlichen Blut. Am Schluß stehen einige Beiträge, welche die früheren Bände ergänzen, so eine Übersicht über Anforderungen an die Verdünnungslösung bei der Hämoglobinbestimmung und Resultate vergleichender Prüfungen verschiedener Hämometer. Der handliche und mit zahlreichen Abbildungen und Diagrammen versehene Band kann jedem an Laboratoriumsfragen interessierten Hämatologen empfohlen werden. H. R. Marti, Basel

Fritz Gramlich: *Die Rezeptorfunktion der Erythrozyten.* Bibl. Haem., Fasc. 25 (Karger, Basel/New York 1966). X + 158 pp., 16 Fig., 24 Tab. Preis sFr/DM 38.—

Die Monographie behandelt Reaktionen der Erythrozytenoberfläche, die unter physiologischen und pathologischen Bedingungen mit Substanzen aus dem umgebenden Plasma erfolgen. Das einleitende Kapitel befaßt sich mit der Erythrozytenmembran und -oberfläche. Dann folgen zwei Kapitel über Reaktionen mit arteigenen und artfremden Substanzen. Die arteigenen sind Hämantikörper und Plasmaproteine; unter den artfremden befinden sich Heterohämolysine, Phytoagglutinine, Fremdproteine, Halbantigene und Mikroorganismen. Die Fortsetzung bilden eigene experimentelle Beiträge zum Problem der Eiweißbeladung der Erythrozytenoberfläche, und das letzte Kapitel enthält Angaben über die verwendete Methodik. Ein Literaturverzeichnis mit 429 Zitaten und ein kurzes Sachregister bilden den Schluß des handlichen und übersichtlich gegliederten Werkes, von dem Prof. Dommeyer in seinem Geleitwort sagt, es sei ungleich komplexer und lasse den ruhelosen und beharrlichen experimentellen Eifer des Autors erkennen. H. R. Marti, Basel

L. Beckman: *Isozyme Variations in Man.* Monographs in Human Genetics, Vol. 1. Editors L. Beckman (Uppsala), M. Hauge (Copenhagen). S. Karger, Basel/New York 1966. 76 p., 33 fig., 22 tab., Preis sF 19.80.

Es ist ein Merkmal der modernen Wissenschaft, daß Entwicklungen einzelner Teilgebiete oft sehr schnell stattfinden als Resultat spezifischer technischer Fortschritte. Die Folge ist ein Strom neuer Literatur, welche selbst für den Spezialisten kaum über-

schbar ist. Das Büchlein stellt den Versuch dar, die hauptsächlichsten neuen Befunde auf dem Gebiet der multiplen molekularen Formen von Enzymen, d. h. Isoenzymen oder Isozymen beim Menschen während der letzten paar Jahre zusammenzustellen. Die Bibliographie ist vom Autor bewußt nicht vollständig gehalten, enthält aber eine Vielzahl von Publikationen neuesten Datums.

Esterasen, alkalische und saure Phosphatasen, Leucin-Aminopeptidase (= Arylamidase), Dehydrogenasen (Lactatdehydrogenase, Glucose-6-Phosphatdehydrogenase, 6-Phosphogluconatdehydrogenase) sowie die Phosphoglucomutase und Katalase werden kurz besprochen. Tabellen und schematische Zeichnungen über den Ausfall der Elektrophoresen ergeben ein anschauliches Bild der Hauptbefunde. Der Versuch einer Synthese über die molekulare Heterogenität verschiedener Enzymsysteme folgt am Ende der Publikation. Sie sind spekulativer Natur, aber zeugen von der guten Kenntnis der Materie durch den Autor. Ontogenetische Verschiedenheiten, chromosomale Lokalisationen von Enzymgenen, hybride Enzyme sowie quantitative Variationen in Serum und Gewebe kommen zur Besprechung. Das Buch ist für enzymologisch und genetisch interessierte Fachleute geschrieben. Druck und Ausstattung sind vorzüglich.

U. C. Dubach, Basel

D. J. Weatherall: **The Thalassaemia Syndromes.** Blackwell Scientific Publications, Oxford 1965. 272 S. Preis 45 s.

Es ist seit langem bekannt, daß die Thalassämie keine einheitliche Anomalie darstellt. Die vorliegende Monographie bringt eine sehr willkommene Zusammenstellung der heutigen Kenntnisse über Pathogenese und Klinik der verschiedenen Thalassämie-Formen. Nach einer kurzen historischen Übersicht folgen einleitend leicht verständliche Kapitel über die genetische Kontrolle der Hämoglobinsynthese und die genetischen Grundlagen der Thalassämien. Dann werden die klassischen und die atypischen Formen der β-Thalassämie, ihre Kombination mit anomalen Hämoglobinen und die Hämoglobin Lepore-Syndrome besprochen. Auf analoge Weise wird die α-Thalassämie abgehandelt. Ein spezielles Kapitel ist dazwischen der hereditären Hämoglobin-F-Persistenz gewidmet. Der folgende Teil des Buches behandelt rein klinische Probleme: klinische Symptomatologie, diagnostische Methoden und Therapie. Am Schluß steht schließlich ein Kapitel über die der Thalassämie zugrundeliegenden Defekte der Hämoglobinsynthese und die heute zur Diskussion stehenden pathogenetischen Mechanismen. Die ausgezeichnete Monographie ist leicht lesbar, übersichtlich gegliedert und enthält zahlreiche gute, instruktive Bilder. Jedes Kapitel ist mit einer Zusammenfassung versehen und am Schluß des Buches findet man eine reichhaltige Literaturzusammenstellung und ein nützliches Schlagwortverzeichnis. Der Autor, der viele eigene Untersuchungsergebnisse mit verwerten konnte, ist von früheren Arbeiten her als kompetenter Fachmann für Fragen der Thalassämien international bekannt. Das Buch kann allen Hämatologen in Klinik und Laboratorium empfohlen werden.

H. R. Marti, Basel

G. Winqvist: **Comparative Leukaemia Research.** Proceedings of an International Wenner-Gren Symposium held in Stockholm, September 1965. Pergamon Press, Oxford 1966. 268 S.

Das vorliegende Buch gibt die verschiedenen Referate anläßlich des Internationalen Wenner-Gren-Symposiums in Stockholm wieder. Das Hauptgewicht wird dabei auf die Virusätiologie der experimentellen Leukämien gelegt. Hierauf kommt auch die Epidemiologie der Human-Leukämie zur Sprache. Interessant sind die verschiedenen Übersichten über die bisher beobachteten Tier-Leukämien. Jedem, der an der Leukämieforschung interessiert ist, und speziell demjenigen, der sich mit der Virusätiologie der Leukosen abgibt, vermittelt dieses gedruckt vorliegende Symposium, das auch die jeweilige an die Vorträge sich anschließende Diskussion enthält, wertvolle Einblicke und Anregungen.

S. Moeschlin, Solothurn

D Metcalf The Thymus (Recent Results in Cancer Research / Fortschritte der Krebsforschung / Progrès dans les recherches sur le cancer vol. V) Springer Verlag, Berlin/Heidelberg/New York 1966. 44 figures, 144 pages, 8 vo. Cloth DM 24 –

Die experimentelle Thymusforschung hat seit der Entdeckung der immunologischen Wirkungen der neonatalen Thymektomie im Jahre 1961 einen enormen Umfang angenommen. In der vorliegenden Monographie gibt Donald Metcalf einer der besten Kenner des Gebietes, einen umfassenden und kritischen Überblick über das bisher Bekannte. Nach einem einleitenden Kapitel über die Struktur des Thymus werden die verschiedenen Möglichkeiten der Thymustransplantation diskutiert. Im folgenden Kapitel, das sich mit der Rolle des Thymus bei der Lymphocytenbildung befaßt, wird darauf hingewiesen, daß zwar im Thymus reichlich Lymphocyten gebildet werden, die aber nach kurzer Zeit zugrunde gehen. Nur ein kleiner Teil wandert in die übrigen lymphatischen Organe des Körpers aus. Viel wichtiger als die Lymphocytenemigration ist die vom Autor entdeckte Regulation der Lymphopoese durch humorale Thymusfaktoren. Diese Faktoren werden deshalb ausführlich diskutiert. Anschließend wird die Bedeutung des Thymus bei immunologischen Reaktionen und bei der Immuntoleranz behandelt und die Beziehung der Thymusdrüse zu Autoimmunkrankheiten erörtert. Die immunologischen Folgen der neonatalen Thymektomie unterscheiden sich nicht prinzipiell von denjenigen der Thymektomie beim Erwachsenen, besonders bei gleichzeitiger Ganzkörperbestrahlung. Es ist schon längere Zeit bekannt, daß die Thymektomie die Entwicklung von lymphatischen Leukaemien bei Mäusen und Ratten verhindern kann. Die möglichen Wirkungsmechanismen dieser Vorgänge werden erörtert. Abschließend wird noch auf die Beziehung zwischen Thymusdrüse und Karzinomentwicklung hingewiesen. Die außerordentlich klar und allgemein verständlich abgefaßte Monographie von Donald Metcalf kann allen denjenigen, die sich über die neuesten Kenntnisse auf diesem faszinierenden Gebiet orientieren möchten, sehr empfohlen werden.

F Gloor, Basel

UICC Monograph Series vol. 4 Cancer Detection. Prepared by the Cancer Detection Committee of the Commission on Cancer Control. Verlag Springer Berlin 1967 VIII + 82 S., 10 Abb., Preis DM 28.–

Diese Monographie der Unio Internationalis contra Cancrum (UICC) umschreibt den Aktivitätsbereich des UICC-Komitees für Krebsfrüherfassung (Cancer Detection). Gleichzeitig enthält sie eine Zusammenfassung der Tätigkeit dieses internationalen Komitees während der Jahre 1963 bis 1965 und eine nach Ländern geordnete Liste der bestehenden nationalen Programme für die Frühdiagnose des Krebses. Das Komitee befaßt sich mit der Organisation solcher Programme sowie mit der Registrierung der verschiedenen Krebsarten und präkanzerösen Zustände. Das Ziel ist eine Verminderung der Krebsmorbidität und -mortalität durch möglichst frühzeitiges Erfassen der Krankheit.

Das leicht zu lesende Buch beschreibt auf knappem Raum (82 Seiten) nacheinander die geographischen Verschiedenheiten von Krebshäufigkeit und -mortalität, den theoretisch erreichbaren und praktisch erreichten Effekt der Krebsdiagnose beim symptomlosen Patienten, die Methoden der Krebsfrühentdeckung (nach Lokalisationen geordnet) und die Rolle der Publikumsaufklärung und -erziehung bei der Früherfassung des Krebses. Wertvolle bibliographische Hinweise schließen die Monographie ab.

Immer mehr werden auch bei uns – sowohl in Ärztekreisen als auch von Politikern und in der Öffentlichkeit – Fragen der Aufklärung der Bevölkerung, des Wertes der Gesundenuntersuchung und der Möglichkeiten der Krebsverhütung diskutiert. Solche Diskussionen sollten sich, um fruchtbar zu sein, möglichst wenig auf affektive Momente oder persönliche Meinungen, dagegen möglichst viel auf wissenschaftlich gesicherte

Grundlagen und statistisch ausgewertete Erfahrungen stützen. Die vorliegende Monographie bildet eine ausgezeichnete Einführung in diese Probleme. Sie vermittelt eine gute Übersicht über die heute bekannten Methoden der Früherfassung und die damit bisher erreichten Resultate.

G. Martz, Zürich

F. Koller, F. Duckert and F. Streuli: **Pathogenesis and Treatment of Thromboembolic Diseases.** International Symposium, Basel August 29—September 1 1963. Verlag Schattauer, Stuttgart 1966. XX + 583 S., 163 Abb., 86 Tab. Preis DM 68,–

Ausgezeichneter Überblick über die Pathogenese und Behandlung von Thromboembolien. Nach einleitenden Bemerkungen von Koller wird die pathologische Anatomie der Thrombose in drei Referaten behandelt und diskutiert. Interessante Referate über die Virchowsche Trias eröffnen das Kapitel Pathogenese, in dem die Rolle der Thrombozyten für die Thromboseentstehung besonders eingehend besprochen wird. Es folgt ein interessantes Referat über die Pharmakologie der peroralen Antikoagulantien und die Wirkung der Antikoagulation bei der Prophylaxe von Venenthrombose und Lungenembolie. Indikation und Kontraindikation der Antikoagulation bei koronarer Herzkrankheit werden eingehend diskutiert. Alle Autoren empfehlen, Patienten mit akutem Herzinfarkt zu antikoagulieren. Drei vergleichende Untersuchungen neuesten Datums (Veteran's administration, Utrecht, Leiden) belegen die Berechtigung der Langzeit-Antikoagulation bei Patienten mit durchgemachten Herzinfarkten. Nach einer Einleitung zur *Untersuchungstechnik* besprechen drei Arbeiten *Indikation* und Resultate der Antikoagulantien-Therapie bei medizinisch und chirurgisch behandelten Patienten mit Gliedmassenarterienverschluß. Von besonderem Interesse ist eine vergleichende arteriographische Studie, wo in der nicht antikoagulierten Kontrollgruppe innert drei Jahren 7,5mal mehr neue Gliedmassenarterienverschlüsse auftraten als in der antikoagulierten Gruppe.

Die folgenden Abschnitte besprechen die Differentialdiagnose cerebro-vaskulärer Affektionen und die Behandlung von Hirnembolie und Sinusthrombose. Interessante Ergebnisse über die Prophylaxe von Thromboembolien in Chirurgie, Gynäkologie und Geburtshilfe folgen. Das Buch schließt mit einer ausführlichen Diskussion von Technik und Indikation der thrombolytischen Therapie und des Syndroms der allgemeinen intravaskulären Gerinnung. Das Buch sei jedem praktizierenden Arzt und jedem Kliniker empfohlen. Es ist eine Fundgrube neuer Erkenntnisse und bietet wertvollste Informationen über Theorie und klinische Probleme der Thromboembolie.

L. K. Widmer, Basel

P. L. Mollison: **Blood Transfusion in Clinical Medicine.** 4th edition. 863 S., Blackwell, Oxford 1967

Nach 6 Jahren ist dieses Standardwerk der Transfusionskunde in vierter Auflage erschienen. Mollison hat es dabei gründlich überarbeitet. Besonders wertvoll und von eigener experimenteller Arbeit geprägt ist die Darstellung über die Immunchemie der Blutgruppenantikörper und die quantitative Erfassung blutgruppenserologischer Antigen-Antikörperreaktionen. Für den Kliniker bedeutsam sind seine Ausführungen über aktuelle Probleme des Morbus haemolyticus neonatorum, wie die Immunprophylaxe mit Anti-D, der Nachweis von Gallepigmenten in der Amnionflüssigkeit und die Problematik der intrauterinen Transfusion. Wie die früheren Auflagen gehört auch der neue «Mollison» in die Hand eines jeden, der sich mit klinischen Transfusionsproblemen ernsthaft auseinanderzusetzen gedenkt.

A. Hässig, Bern

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Bearbeitet von G. Bonzon, Basel

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